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IVANA MARIA ZACCARA CUNHA ARAÚJO

Influência da fotobiomodulação na acetilação das histonas, viabilidade, migração e diferenciação de células-tronco da polpa dental e na formação radicular de molares de ratos com rizogênese incompleta e necrose pulpar.

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Tese apresentada ao Programa de Pós-Graduação em Odontologia da Faculdade Odontologia da Universidade Federal do Rio Grande do Sul com requisito para obtenção do título de Doutora em Odontologia, área de concentração em Clínica Odontológica/Endodontia.

Orientadora: Profª. Dra. Patrícia Maria Poli Kopper Móra

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BANCA EXAMINADORA

_____
Profª. Dra. Patrícia Maria Poli Kopper Móra - Orientadora

_____
Profª. Dra. Fernanda Visioli - UFRGS

_____
Profª. Dra. Simone Bonato Luisi - UFRGS

_____
Prof. Dr. Carlos Augusto Galvão Barboza – UFRN

_____
Profª. Dra. Daiana Elisabeth Böttcher - PUCRS (Suplente)

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e Rosane por todo amor, paciência e
espera.

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“Happiness is only real when shared.”
(Christopher McCandless, Into the wild, 1996)

RESUMO

O objetivo do estudo foi avaliar, in vitro, a influência da fotobiomodulação (PBM) na, viabilidade e migração, relacionados à acetilação das histonas, e na diferenciação de células-tronco da polpa de dentes permanentes humanos (hDPSCs); e, in vivo, sua influência no reparo radicular de molares de ratos com necrose pulpar e rizogênese incompleta. hDPSCs foram caracterizadas e utilizadas nos experimentos de três artigos científicos. A PBM foi empregada utilizando-se laser diodo InGaAlP (100mW, 660nm, 3J/cm^2, energia total por aplicação 1J em 10s). Em todos os experimentos in vitro os grupos foram divididos de acordo com o uso da PBM e os intervalos de irradiação foram de 6 horas. A viabilidade destas células foi avaliada pelo ensaio de MTT e a migração pelo *scratch.* A acetilação das histonas das hDPSCs foi avaliada por imunofluorescência. Para avaliar o potencial de diferenciação e a deposição de tecido mineralizado, foram utilizados os meios adipogênico, condrogênico e osteogênico, complementado pelo ensaio de atividade ALP, em modelo 3D, em gel de agarose 0,3%. Nos ensaios de diferenciação também foi avaliada a condição nutricional (regular: 10% SFB; ou déficit: 5%SFB). Os resultados do MTT, *scratch* e da acetilação das histonas foram obtidos em até 24 horas e nos ensaios de diferenciação foram analisados em 7 e 14 dias. Para o experimento in vivo, molares inferiores de ratos Wistar foram expostos ao meio bucal por 3 semanas e, após, tratados com pasta antibiótica por uma semana. A seguir, os canais foram irrigados e divididos em 6 grupos (n=6): MTA; coágulo sanguíneo (CS); hDPSC; MTA+PBM; CS+PBM; hDPSC+PBM. Dois grupos não foram expostos ao meio bucal: Dente hígido+PBM (n=6), Dente hígido (controle positivo, n=3); e um grupo foi mantido exposto durante todo o período experimental: Dente necrótico (controle negativo, n=3). Durante 30 dias as irradiações foram feitas com intervalos de 24 horas. Após, os ratos foram eutanasiados e realizou-se avaliação histológica e imunohistoquímica. Os dados obtidos foram tabulados e analisados estatisticamente. A PBM aumentou a viabilidade das células ($P<0.001$). No ensaio de scratch o grupo PBM acelerou o fechamento do ferida até 12 horas ($P<0.001$) e esta fechou em 18 horas, sendo mais rápido que o controle ($P<0.05$). A PBM aumentou a acetilação das histonas ($P<0.001$); Após 14 dias, a PBM intensificou a diferenciação nos três meios empregados e na atividade ALP, comparado com os controles ($P<0.05$). No estudo in vivo, o controle negativo apresentou infiltrado de neutrófilos maior do que os demais grupos ($P<0.05$). Os grupos Controle negativo, Dente hígido e Dente hígido+PBM apresentaram menos condensação fibrosa ($P<0.05$). Todos os grupos formaram mais tecido mineralizado que o controle negativo ($P<0.05$). PBM+MTA, +CS ou +hDPSC formaram mais tecido mineralizado que os grupos não irradiados ($P<0.05$). MTA+PBM induziu apicificação ($P<0.05$). A marcação para BSP confirmou os achados histológicos relacionados a formação de tecido mineralizado e as hDPSCs, com e sem PBM, exibiram maior porcentagem de células positivas para BSP. Conclui-se que a PBM desempenhou papel importante, *in vitro*, aumentando a diferenciação, viabilidade e migração celular que estão relacionados a acetilação das histonas. Além disso, a PBM pode ser uma alternativa de tratamento adjuvante para dentes permanentes com necrose pulpar e rizogênese incompleta favorecendo o reparo radicular.

Palavras-Chave: Células-tronco. Epigenética. Terapia da fotobiomodulação. Dentes permanentes com rizogênese incompleta.

ABSTRACT

The aim of this study was to evaluate, in vitro, the influence of photobiomodulation (PBM) on viability and migration, related to histone acetylation, and differentiation of human dental pulp stem cells (hDPSCs); and, in vivo, its influence on the root repair of rat molars with pulp necrosis and open apex. The hDPSCs were characterized and used in the experiments of three scientific papers. The PBM was applied using InGaAlP diode laser (100mW, 660nm, 3J / cm^2, total energy per application 1J in 10s). In all in vitro experiments, the groups were divided according to the use of PBM, with 6-hour irradiation intervals. The viability of these cells was assessed by MTT assay and migration by scratch. The histone acetylationof hDPSCs was evaluated by immunofluorescence. To evaluate the potential for differentiation and deposition of mineralized tissue, adipogenic, chondrogenic and osteogenic mediums were used, complemented by the ALP activity assay in 3D model, in 0.3% agarose gel. In differentiation assays, the nutritional status was also evaluated (regular: 10% FBS; or deficit: 5% FBS). The MTT, scratch and histone acetylation results were obtained until 24 hours, and the differentiation assays were analyzed at 7 and 14 days. For the in vivo experiment, lower molars of Wistar rats were exposed to oral environment for 3 weeks and then treated with antibiotic paste for one week. Next the root canals were irrigated and divided into 6 groups (n=6): MTA; BC (blood clot); hDPSC; healthy tooth+PBM; MTA+PBM; BC+PBM; hDPSC+PBM. In hDSPC groups, 1% agarose gel scaffold was used for cell support. Two groups were not exposed to the oral environment: healthy tooth+PBM (n=6), healthy tooth (positive control, n=3); and one stayed exposed during the role experimental period: necrotic tooth (negative control, n=3). For 30 days the irradiations were done at 24-hour intervals. Thereafter, the rats were euthanized and histological and immunohistochemical evaluations were performed. Data were tabulated and statistically analyzed. PBM increased cell viability ($P<0.001$). In the scratch assay, the PBM group accelerated wound closure up to 12 hours ($P<0.001$) and closed at 18 hours, being faster than the control ($P<0.05$). PBM increased histone acetylation ($P<0.001$). After 14 days, PBM improved the differentiation in the three mediums employed and the ALP activity, compared to the controls ($P<0.05$). In the in vivo study, the negative control had greater neutrophil infiltrate than the other groups ($P<0.05$). Negative Control, Healthy tooth and Healthy tooth+PBM groups presented less fibrous condensation ($P<0.05$). All groups formed more mineralized tissue than the negative control ($P<0.05$). PBM+MTA, +BC or +hDPSC formed more mineralized tissue than non-irradiated groups ($P<0.05$). MTA+PBM induced inoculation ($P<0.05$). BSP labeling confirmed the histological findings related to mineralized tissue formation and hDPSCs, with and without PBM, exhibited a higher percentage of BSP-positive cells. It is concluded that PBM played an important role, *in vitro,* increasing differentiation, viability and cell migration that are related to histone acetylation. Moreover, the PBM may be an adjutant treatment alternative for permanent teeth with pulp necrosis and open apex, favoring root repair.

Keywords: Stem cells. Epigenetic. Photobiomodulation therapy. Permanent teeth with open apex.

SUMÁRIO

1 INTRODUÇÃO

1.1 CARACTERÍSTICAS GERAIS DAS CÉLULAS-TRONCO DA POLPA DENTAL (DPSC)

Células-tronco (CTs) adultas multipotentes são encontradas em tecidos já desenvolvidos do feto, recém-nascido, jovem e adulto (KRABBE, ZIMMER, MEYER, 2005; SERAKINCI, KEITH, 2006) e são caracterizadas pela capacidade de auto-renovação e diferenciação celular (GRONTHOS et al., 2000). As terapias com células-tronco já provaram ser bem sucedidas sendo eficazes em transplantes de células hematopoiéticas de medula óssea, regeneração da pele com cultivo de células progenitoras ou tratamento de córnea danificada (RAMA et al., 2010; BIANCO et al., 2014).

As células-tronco embrionárias apresentam a habilidade de proliferar em cultura sem perder sua capacidade de diferenciação em tipos celulares específicos, tornando-se uma poderosa ferramenta de pesquisa (LO, PARHAM, 2009). Porém, sua utilização em pesquisas envolve conflitos éticos, religiosos, políticos e legais (LO, PARHAM, 2009). Em contrapartida, as células adultas multipotentes têm a capacidade de renovar-se e diferenciar-se em diversas linhagens de células e podem ser obtidas a partir de muitos tecidos, como por exemplo, a medula óssea (CAPLAN, 1991), o tecido adiposo (SECCO et al., 2008), o cordão umbilical (VIEIRA et al., 2008) e a polpa dentária (GRONTHOS et al., 2000; SHI, ROBEY, GRONTHOS, 2001; MIURA et al., 2003; DE MENDONÇA COSTA et al., 2008).

A Sociedade Internacional de Terapia Celular (ISCT), em 2006, propôs um painel de marcadores de superfície celular para identificação de CTs em que a população de células avaliadas deve apresentar pelo menos 95% de positividade para os seguintes marcadores: CD73, CD90, CD44, CD146 e CD105; e negativas para CD34 (células endoteliais e progenitores hematopoiéticos), CD45 (leucócitos), CD11b ou CD14 (monócitos/ macrófagos), CD19 ou CD79α (linfócitos B), e HLA-DR (células apresentadoras de antígenos). Além disso a capacidade de diferenciação das CTs em osteoblastos, condroblastos e adipócitos em condições de cultivo celular representa outro método importante que caracteriza as CTs (CHEN et al., 2006; DOMINICI et al., 2006). As CTs têm se destacado na medicina regenerativa onde a taxa de proliferação e o sucesso da diferenciação de células transplantadas

representa um grande avanço para engenharia de tecidos (CAPLAN, 2005; LEONIDA, et al., 2013).

Na área da Odontologia, as CTs têm sido isoladas de diferentes porções do complexo dento-alveolar, como as células do folículo dentário – HDFC (MORSCZECK et al., 2005; KÉMOUN et al., 2007), as células do ligamento periodontal - PDLSC (SEO et al., 2004; GRONTHOS et al., 2006; VASCONCELOS et al., 2011; SOARES et al., 2015), as células da papila dental – SCAP (SONOYAMA et al., 2008; HUANG et al., 2008), a polpa dentária de dente decíduo – SHED (MIURA et al., 2003; KERKIS et al., 2006) e a polpa dentária de dente permanente – DPSC (GRONTHOS et al., 2000, 2002; SHI, ROBEY, GRONTHOS, 2001; DE MENDONÇA COSTA et al., 2008; ZACCARA et al., 2015).

Gronthos et al. (2000) identificou pela primeira vez células-tronco derivadas da polpa de dente permanente e, a partir disso, passou-se a utilizar o termo DPSCs (*dental pulp stem cells*) para referir-se a elas. Estas células apresentaram alta capacidade proliferativa e de diferenciação, expressão de diversos marcadores de células da medula óssea e neurais, além de apresentarem morfologia fibroblastóide, semelhante às células-tronco embrionárias.

Posteriormente diversos estudos foram realizados para avaliar as DPSCs e foi observado que estas células mantém potencial altamente proliferativo, mesmo após subcultivo extensivo, e apresentam capacidade de induzir formação óssea in vivo (GRONTHOS et al., 2002; SHI, ROBEY, GRONTHOS, 2001; NAKASHIMA, AKAMINE, 2005). Para tanto, é necessário um meio indutor apropriado e um arcabouço para induzir a formação de osso, cemento e dentina (BATOULI et al., 2003; SHI et al., 2005). Além disso, sugere-se que as DPSCs estão envolvidas na indução da formação de osso durante a erupção de dentes permanentes (MIURA et al., 2003). Diante das propriedades apresentadas pelas DPSCs, elas têm sido estudadas para diversos propósitos regenerativos, não só na Odontologia, como também em várias aplicações na medicina (DE MENDONÇA-COSTA et al., 2008; KERKIS et al., 2006).

1.2 REGULAÇÃO EPIGENÉTICA DAS CÉLULAS-TRONCO (CTs)

As estratégias regenerativas, com base nas propriedades das CTs, direcionaram a atenção dos estudos para a identificação de reguladores celulares que controlam a proliferação e diferenciação celular (DUNCAN et al., 2016).

Todas as células diferenciadas no organismo possuem o mesmo material genético, pois originaram-se de uma mesma célula-tronco totipotente. Entretanto, apresentam padrão de expressão gênica específico para cada tipo celular, devido a mecanismos de silenciamento ou ativação gênica de determinados tecidos específicos (LI, 2002).

A regulação epigenética refere-se a processos biológicos que regulam alterações mitóticas ou meióticas hereditárias na expressão de genes sem alterar a sequência de DNA (WU, SUN, 2006). Os principais mecanismos epigenéticos observados em muitas linhagens, incluindo a polpa dentária, são: a metilação da citosina do DNA; as modificações das histonas, como a acetilação e a metilação das caudas das histonas; e, em pequena proporção, o RNA não codificado que controla a regulação pré e pós-transcricional da expressão gênica (WU, SUN, 2006; GOPINATHAN et al., 2013; SEO et al., 2015).

Para melhor entendimento dos mecanismos epigenéticos, torna-se necessário compreender a composição da cromatina que é formada pelo complexo de DNA e proteínas presentes no núcleo de células eucariotas, onde as principais proteínas são da família das histonas. A unidade básica da cromatina denomina-se nucleossomo, composto por 147 pares de base de DNA organizados ao redor de duas copias de cada histona H2A, H2B, H3 e H4, formando um octâmero, enquanto a histona H1 se liga aos nucleossomos adjacentes, "empacotando-os" (LUGER, RICHMOND, 1998).

Cada histona apresenta um domínio longo e flexível (caudas), que se projeta do nucleossomo. Nessas caudas ocorrem a maioria das modificações das histonas, como acetilação de lisina, fosforilação de serina, metilação de lisina, metilação de arginina e ubiquitinação de lisina (GOODSELL, 2003; SONG, HAN, BANG, 2011; RANDO 2012). Além disso, a acetilação de histonas, no resíduo lisina 9 da histona H3, é a modificação mais comum associada à transcrição ativa, aumentando significativamente a expressão gênica. Portanto, a lisina 9 da histona H3 é considerada um marcador epigenético (THIAGALIGAM et al., 2003).

A organização da cromatina permite ou dificulta a transcrição genética devido ao acesso dos fatores da transcrição (GOODSELL, 2003). O grau de acetilação ou desacetilação da histona, presente na composição da cromatina, influencia sua organização e está correlacionado com o nível de transcrição (SCHÜBELER et al., 2004). A modificação pós-traducional mais estudada é a acetilação que está relacionada com a cromatina desdobrada e geneticamente ativa (GOODSELL, 2003). Ela ocorre pela adição de um grupo acetil aos resíduos de lisina, localizados nas caudas das histonas, realizada pela enzima histona acetiltransferase (HAT), que causa neutralização das cargas positivas desses resíduos de lisina, enfraquecendo as interações eletrostáticas entre as histonas e o esqueleto de fosfato do DNA, carregado negativamente, desatrelando o DNA da histona, ou seja, tornando a cromatina mais exposta aos fatores de transcrição, facilitando a expressão proteica (ZUPKOVITZ et al., 2006). Em contrapartida, a remoção do grupo acetil, realizada pela enzima histona desacetilase (HDAC), leva ao aumento das interações entre as histonas e o DNA, gerando uma maior compactação das estruturas nucleossômicas, limitando a atividade gênica e dificultando a ligação dos fatores de transcrição e, assim, a expressão proteica (DE RUIJTER et al., 2003).

Atualmente, estudos têm demonstrado que muitos processos biológicos, incluindo proliferação, migração e diferenciação celular, são regulados por mecanismos epigenéticos modulados pelo equilíbrio das enzimas HDAC e HAT (LEWIS et al., 2014). Dessa forma, torna-se interessante identificar mecanismos com potencial de regular positivamente eventos epigenéticos (DUNCAN et al., 2011).

A regulação epigenética relacionada à regeneração e reparo da polpa têm sido estudada avaliando os inibidores das histona desacetilases (HDACi), que proporcionam aumento da transcrição genética (DUNCAN et al., 2011, 2013, 2016; HUI et al., 2017). Tal fato demonstra uma perspectiva para aplicação destes inibidores em algumas situações clínicas da área da Endodontia (PAINO et al., 2014; SEO et al., 2015, DUNCAN et al., 2016). Neste sentido, vários tipos de HDACi foram avaliados, incluindo tricostatina A (TSA), ácido valproico (VPA) e ácido butírico, e mostraram alterar reversivelmente a acetilação de histonas, promovendo a transcrição gênica, induzindo a proliferação, diferenciação e efeitos antiinflamatórios em CTs (JIN et al., 2013; MARKS, 2010; SHUTTLEWORTH, BAILEY, TOWNSEND,

2010; MAHMUD et al., 2014, DUNCAN et al., 2016). No entanto, a influência da fotobiomodulação (PBM) na regulação epigenética ainda não foi estabelecida.

1.3 TERAPIA DE FOTOBIOMODULAÇÃO (PBM)

"Terapia de fotobiomodulação" é o termo mais recente para a terapia de aplicação de laser de baixa intensidade nos dados da Medical Subject Headings (MeSH), sistema em língua inglesa que diz respeito à nomenclatura médica, baseando-se na indexação de artigos no campo das ciências da saúde de acordo com o sistema MEDLINE-PubMed. Alguns outros nomes foram utilizados anteriormente para esta terapia, incluindo terapia com laser (ou luz) de baixa intensidade (*low-level laser therapy* - LLLT), irradiação com laser de baixa intensidade (*low-level laser irradiation* - LLLI), terapia com laser de baixa potência, cold laser, laser soft, demonstrando que existe claramente uma falta de consistência e consenso sobre a terminologia (ANDERS, LANZAFAME, ARANY, 2014). O termo mais utilizado é terapia com laser de baixa intensidade (LLLT), sendo frequentemente mencionado no MeSH. Porém, este termo é ambíguo, pois as palavras "baixa" e "intensidade" são vagas e não definidas com precisão, enquanto a palavra "laser" não é mais apropriada, pois outros tipos de dispositivos de luz, como LEDs e fontes de luz de banda larga, são usados atualmente para esta aplicação (ARANY et al., 2012).

Anders, Lanzafame e Arany (2015) sugeriram uma definição mais abrangente para terapia de fotobiomodulação: uma terapia de luz que utiliza formas não ionizantes de fontes de luz, incluindo lasers, LEDs e luz de banda larga, no espectro visível e infravermelho, sendo um processo não-térmico que envolve cromóforos endógenos que provocam eventos fotofísicos e fotoquímicos em várias escalas biológicas. Este processo promove resultados terapêuticos benéficos, incluindo, entre outros, o alívio da dor ou inflamação, imunomodulação e promoção da cicatrização de feridas e regeneração tecidual (ANDERS, LANZAFAME, ARANY, 2015).

O termo terapia de fotobiomodulação é preciso e específico para esta aplicação e seu uso de forma universal reduziria ou eliminaria a confusão no campo da literatura científica e leiga. Isso geraria uma imagem unificada e positiva para mostrar as importantes aplicações clínicas que a terapia de fotobiomodulação pode

oferecer para várias aplicações médicas (ANDERS, LANZAFAME, ARANY, 2015). Desta forma, no decorrer deste estudo, sempre será utilizado o termo terapia de fotobiomodulação (*photobiomodulation* – PBM), independente dos termos empregados nos artigos citados.

Cabe salientar que a PBM quando utilizada em tecidos biológicos, durante a irradiação, a luz pode ser refletida, espalhada, absorvida ou transmitida para os tecidos circundantes (SCHWARZ, 2009). Além disso, essas interações também podem ser influenciadas por várias características específicas do laser, como a saída de energia, o modo de oscilação (onda contínua ou pulsada) ou o modo de aplicação (contato ou sem contato) (SCHWARZ, 2009).

1.3.1 Propriedades da PBM em nível celular

A PBM transmite energia em níveis baixos e, portanto, não emite som, calor, ou vibrações. Suas reações não são térmicas porque não há aumento imediato de temperatura do tecido irradiado (QUICKENDEN, DANIELS, 1993). As pesquisas que abordam o tema do mecanismo de ação da PBM estão relacionadas com a sua influência nas mitocôndrias que desempenham um papel importante na geração de energia e no metabolismo celular, além de promover a integração de sinais entre as organelas e o núcleo (CARNEVALLI et al., 2003; SILVEIRA et al., 2009; GAO, XING, 2009). Neste sentindo, foi avaliada a atividade mitocondrial em células submetidas à PBM e os estudos revelaram uma associação entre o aumento da função mitocondrial com o aumento da capacidade de regeneração e cicatrização de tecidos (KARU; KOLYAKOV, 2005).

Quando a PBM é utilizada, prótons que são absorvidos por fotorreceptores na célula que alteram a atividade biológica (HAWKINS, ABRAHAMSE, 2006; HAWKINS, ABRAHAMSE, 2007). Dessa forma, a PBM intensifica a formação de um gradiente eletromecânico de prótons transmembranar na mitocôndria, aumentando a eficiência da força próton motriz (PMF) o que induz o aumento do cálcio intracelular. Com a utilização da PBM, este cálcio adicional, transportado para o citoplasma, desencadeia a mitose e aumenta a proliferação celular (FRIEDMANN et al., 1991).

Outros dois processos também tem a capacidade de desencadear sinais mitogênicos nas células: (A) a elevação a curto prazo do pH intracelular através da

criação de um gradiente eletromecânico de prótons; (B) o aumento da produção de ATP através da PMF, que ativa alguns transportadores de íons, como a K-ATPase, e, desta forma, o nível de K+ intracelular aumentado causa a diminuição do potencial de membrana e da concentração de Na+ intracelular que influenciam a proliferação celular (FRIEDMANN et al., 1991; EDUARDO et al., 2007).

1.3.2 PBM na Odontologia

Na Odontologia existe uma tendência à incorporação de métodos menos invasivos com a finalidade de minimizar a dor e o desconforto durante e após as intervenções. Por isso, a PBM tem sido usada em diferentes áreas. A PBM apresenta metodologia simples e produz efeitos benéficos para os tecidos irradiados, como: ativação da microcirculação; produção de novos capilares; efeitos anti-inflamatórios e analgésicos; e, estímulo ao crescimento e à regeneração celular em algumas patologias (CAVALCANTI et al., 2011; NAMMOUR, 2012).

Devido a capacidade da PBM de estimular a proliferação e a migração de células como ceratinócitos, células endoteliais e fibroblastos, que são essenciais para a re-epitelização, angiogênese e formação de tecido de granulação, esta terapia tem sido utilizada para estimular cicatrização de feridas (PEPLOW, CHUNG, BAXTER, 2010). Além disso, a sua utilização pode proporcionar uma reparação tecidual rápida e menos dolorosa em estomatite aftosa recorrente, úlceras traumáticas, lesões herpéticas, pênfigo, pericoronarite, líquen plano e disfunção temporomadibular (CARVALHO et al., 2011; SANCHEZ et al., 2012; GAUTAM et al., 2012). Weissheimer et al. (2017) afirmam que a PBM pode ser usada de forma preventiva evitando mucosites orais em pacientes que receberam transplante de células-tronco hematopoiéticas ou diminuindo sua duração e severidade em pacientes que já apresentavam as lesões. De modo geral, em função de resultados satisfatórios alcançados com cultura de células, na medicina tem sido discutida a capacidade da PBM em contribuir com a regeneração óssea e consolidação de fraturas, prevenção de osteoporose, reparação de nervos e músculos esqueléticos após lesão (FERRARI et al., 2011; YAZDANI et al., 2012).

Atualmente na área da Endodontia, existe uma linha pesquisa que analisa o mecanismo de ação da terapia fotodinâmica (PDT) que representa a associação entre a PBM e fotossensibilizadores exógenos (FS) para promover a desinfecção de

canais radiculares. A PBM, quando utilizada isoladamente, não é capaz de ter ação antimicrobiana; porém, juntamente com FS desencadeia uma cascata de eventos fotoquímicos, resultando na produção de espécies reativas de oxigênio, que são tóxicas para as células tumorais, fungos e bactérias, especialmente para o *Enterococcous. faecalis* (KONOPKA, GOSLINSKI, 2007; TRINDADE et al. 2015).

Entretanto, o presente estudo não teve como objetivo avaliar a ação antimicrobiana da PBM e sim sua interferência na regulação epigenética, proliferação e diferenciação. Além disso, teve como objetivo investigar a capacidade da PBM de estimular a resposta tecidual em situações de dentes com necrose pulpar e rizogênese incompleta.

1.3.3 PBM na proliferação e diferenciação celular

A PBM promove efeitos bioestimuladores e biomoduladores in vivo e in vitro, estimulando o crescimento, aumentando o metabolismo e melhorando a regeneração celular, provocando uma resposta tecidual e induzindo a diferenciação em tipos celulares específicos(KNEEBONE, CNC, FIAMA, 2006; TUBY, MALTZ, ORON, 2007; HOU et al., 2008; MVULA et al., 2008). Estes processos dependem de moléculas como a proteína quinase c (PKC), proteína quinase B (Akt / PKB), Src tirosina cinases e interleucina-8 / 1ª (IL-8 / 1a) (DE FREITAS, HAMBLIN, 2016).

A interação da PBM com as células e os tecidos pode levar a diferentes resultados, dependendo provavelmente de fatores relacionados ao tipo de célula irradiada, as características fisiológicas das células no momento da irradiação e de parâmetros relacionados ao laser, como comprimento de onda, densidade de energia, potência, número de pontos irradiados, diâmetro do feixe, tempo e número de irradiações (ALGHAMDI, KUMAR, MOUSSA, 2012).

Revisões sistemáticas demonstraram que resultados positivos de proliferação e diferenciação relacionados com a PBM foram alcançados quando o comprimento de onda variou entre 600 a 700nm com a densidade de energia variando entre 0,5 a 4 J/cm^2, utilizando maior potência e curto tempo de exposição (ALGHAMDI, KUMAR, MOUSSA, 2012; EMELYANOV, KIRYANOVA, 2015, GINANI et al., 2015). Além disso, o uso de uma densidade de energia superior a 10 J/cm^2 pode danificar os fotorreceptores, reduzindo o efeito fotobiomodulador devido à inibição do metabolismo e consequentemente morte celular (KARU, 1989;

ALGHAMDI, KUMAR, MOUSSA, 2012). A PBM exerce um efeito dose-dependente em respostas biológicas e parece ter um efeito cumulativo em cada nova dose aplicada (HUANG et al., 2009). Outros estudos verificaram que há uma tendência da PBM de aumentar a viabilidade das CTs sem efeitos deletérios (BORZABADI-FARAHANI, 2016; MARQUES et al., 2016). No entanto, considerando a falta de padronização dos parâmetros utilizados e as evidências limitadas, é difícil obter-se uma conclusão clara e comparar os resultados dos diferentes estudos com maior precisão (GINANI et al., 2015; MARQUES et al., 2016).

A este respeito, vários outros estudos têm confirmado que a PBM melhora a proliferação de diferentes tipos de células-troncos (ALMEIDA-LOPES et al., 2001; TUBY, MALTZ, ORON, 2007; STEIN et al., 2008; MVULA et al., 2008; EDUARDO et al., 2008; HORVA´T-KARAJZ, BALOGH, 2009) e apresenta efeito bioestimulador sobre a diferenciação de células-tronco em células formadoras de osso e na indução de deposição de tecido mineralizado com a utilização de biomateriais (ABRAMOVITCH-GOTTLIB et al., 2005; STEIN et al., 2008; HOU et al., 2008).

Considerando que a PBM é efetiva na proliferação de diferentes linhagens de células-tronco, Eduardo et al. (2008) foram os primeiros a submeter hDPSC à PBM. Para avaliar o efeito da PBM nas hDPSCs, os autores empregaram o teste MTT que baseia-se na relação direta entre viabilidade celular, aumento das funções celulares e na produção de ATP, mediados por fotorreceptores nas mitocôndrias (KARU; KOLYAKOV, 2005). Os autores verificaram uma maior atividade proliferativa de DPSCs humanas submetidas à PBM (InGaAlP) de 660nm, densidade de energia de 3J/cm^2 e 20 ou 40 mW (EDUARDO et al., 2008).

Apesar da importância do conhecimento obtido a partir de estudos que avaliaram diferenciação em modelo de cultura monocamada, é importante salientar que eles não simulam a condição do tecido natural (CORDEIRO et al., 2008), limitando sua aplicação e relevância clínica (EDMONDSON, 2014; BASSO et al., 2016; SILVA et al., 2016). A análise de cultura celular tridimensional (3D), pode simular melhor as condições celulares in vivo, tornando-se fisiologicamente mais relevante e preditiva, apresentando maior estabilidade do que culturas celulares em monocamada (SILVA et al., 2016). Desta forma, a cultura 3D é capaz de promover um melhor conhecimento sobre as funções celulares in vivo (VIDYASEKAR et al., 2016), sendo mais adequada para uso em testes de bioatividade (SILVA et al., 2016; THEOCHARIDOU et al., 2016). Entre os vários modelos de cultura 3D, o gel de

agarose (um polissacarídeo de D-galactose e 3,6-anidro-L-galactopiranose derivado das paredes celulares de algas vermelhas) apresenta características favoráveis como ser bioinerte e não tóxico (AURAND, LAMPE, BJUGSTAD, 2012; PAKULSKA, BALLIOS, SHOICHET, 2012; SUZAWA et al., 2015). Além disso, uma das propriedades importantes da agarose é que sua rigidez pode ser alterada, permitindo a modificação das propriedades mecânicas da cultura 3D (ANNIBALLI et al., 2013).

1.3.4 Associação dos princípios da engenharia de tecidos com a PBM para o tratamento de dentes permanentes com rizogênese incompleta e necrose pulpar

A ocorrência de necrose pulpar em dentes permanentes com rizogênese incompleta representa uma situação clínica desafiadora, pois quando isso ocorre a formação radicular é interrompida, deixando-os susceptíveis a fratura, pois as paredes dentinárias estão finas e curtas (HARGREAVES, DIÓGENES, TEIXEIRA, 2013).

Alguns protocolos de tratamento já descritos favorecem a manutenção da viabilidade de células-tronco presentes na região da papila apical (HUANG et al., 2008; SONOYAMA et al., 2008). Desta forma, torna-se possível a realização da apicigênese em dentes que seriam tratados com procedimentos que visam a apicificação, onde não ocorre o desenvolvimento completo da raiz dentária, deixando a estrutura radicular frágil e com risco de fratura (SIMON et al., 2007; COTTI, MEREU, LUSSO, 2008; JUNG, LEE, HARGREAVES, 2008; JEERUPHAN et al., 2017). Além disso, estudos em animais mostram que, nos casos em que realizam-se protocolos que estimulam a apicigênese, os tecidos que se formam são semelhantes ao osso ou cemento (WANG et al., 2010; YAMAUCHI et al., 2011).

O tratamento ideal para dentes permanentes imaturos necrosados seria o desenvolvimento de procedimentos biológicos para reposição de tecidos danificados, tal como a regeneração do tecido dentinário, promovendo a manutenção do dente e o desenvolvimento radicular normal (WANG et al., 2013). Para isso, torna-se necessário, além de um diagnóstico preciso, o conhecimento dos processos biológicos através de pesquisas envolvendo os três princípios da engenharia de tecidos: células-tronco, biomateriais (*scaffolds*) e fatores de

crescimento (HARGREAVES, DIOGENES, TEIXEIRA, 2013; SCHMALZ, SMITH, 2014).

O primeiro princípio diz respeito a utilização de células-tronco capazes de se diferenciar no componente de tecido desejado (HARGREAVES, DIÓGENES, TEIXEIRA, 2013). O uso de DPSCs é de grande interesse para a engenharia de tecidos, devido à facilidade de isolamento e expansão em cultura (GRONTHOS et al., 2000). Além disso, tais células apresentam propriedades de proliferação, de diferenciação e capacidade imunossupressora, suprimindo a resposta imune das células T in vitro e in vivo (ABUMAREE et al., 2012). Tal capacidade imunossupressora permite que as células-tronco de uma determinada espécie possam ser transplantadas para outras (PIERDOMENICO et al., 2005).

No entanto, as células, quando transplantadas para outro ambiente, precisam de uma estrutura para suportar e fornecer sua viabilidade, desta forma, os biomateriais (*scaffolds)*, segundo princípio da engenharia de tecidos, desempenham um papel fundamental, fornecendo um modelo tridimensional para promover neoformação (VAN HOUT et al., 2011). Entretanto, não existe um modelo de estrutura único que seja aplicável a todas as situações, tornando-se necessária a pesquisa e o desenvolvimento de materiais para diferentes situações de regeneração (SITTINGER, HUTMACHER, RISBUD, 2004). Um *scaffold* biocompatível deve ser usado para prevenir reações adversas do tecido, fornecer uma superfície que facilite a adesão, proliferação e organização espacial das células (HARGREAVES, DIOGENES, TEIXEIRA, 2013).

A escolha do *scaffold* para o tratamento de dentes com necrose pulpar e rizogênese incompleta ainda é controversa e, nesta situação clínica, o *scaffold* deve apresentar capacidade de controle da contaminação do canal radicular, controlar e suportar a vascularização ao longo da raiz, permitir a diferenciação de odontoblastos e suportar a formação de tecido mineralizado (GALLER et al., 2011). Alguns materiais já foram utilizados para analisar a regeneração do complexo dentina-polpa, tais como: HA/TCP, Gelfoam, polímero sintético (PLG), hidrogel sintético, colágeno tipo I e III, coágulo sanguíneo, plasma rico em plaquetas (PRP) e quitosano (MIURA et al., 2003; WANG et al., 2013; HUANG et al., 2010; IOHARA et al., 2011; ZHU et al., 2012; MANGIONE et al., 2017; PALMA et al., 2017). Entre os vários modelos de *scaffolds* para implantação em animais, o gel de agarose (um polissacarídeo de D-galactose e 3,6-anidro-L-galactopiranose derivado das paredes celulares de algas

vermelhas) apresenta, além das propriedades já citadas, potencial de diminuição da rejeição imune em ratos, além de ser barato, evitando a utilização de *scaffolds* sintéticos caros e com longo tempo de preparo (AURAND, LAMPE, BJUGSTAD, 2012, PAKULSKA, BALLIOS, SHOICHET, 2012, SUZAWA et al., 2015; ABUELBA et al., 2015). O gel de agarose já foi avaliado em combinação com células-tronco para gerar uma variedade de tecidos, incluindo osso, cartilagem e células neurais (AWAD et al., 2004; SÁNCHEZ-SALCEDO et al., 2008; SUZAWA et al., 2015). No entanto, até o presente momento não foi testado como *scaffold* para suportar células-tronco transplantadas em canais radiculares de ratos.

De acordo com a engenharia de tecidos, o terceiro princípio importante para alcançar o sucesso é a presença de fatores de crescimento (HARGREAVES, DIOGENES, TEIXEIRA, 2013). De acordo com alguns estudos, o coágulo sanguíneo é um útil carregador de fatores de crescimento e diferenciação para gerar tecido semelhante a polpa no espaço do canal anteriormente necrótico (MAEDA et al., 2004; THIBODEAU, TROPE, 2007). Os fatores de crescimento estão presentes no coágulo sanguíneo devido a sua origem do componente vascular do tecido periapical (MAEDA et al., 2004; THIBODEAU, TROPE, 2007; ZHU et al., 2012). Além disso, foi observado por Galler et al. (2016) que o condicionamento da dentina com EDTA, durante a irrigação final do canal, proporciona diferenciação celular, expressando DSPP (sialoproteína dentinária), com a consequente liberação de fatores de crescimento das paredes dentinárias que se direciona para o biomaterial presente dentro do canal radicular.

Os procedimentos biológicos para a apicigênese e a reposição de tecidos danificados requerem muito tempo, além disso, as células-tronco usadas para protocolos regenerativos devem ser mantidas viáveis por tempo suficiente para que a formação de tecido ocorra (TROPE et al., 2002). Desta forma, devido ao tempo prolongado, falhas ou fratura da raiz podem ocorrer (TROPE et al., 2002). Sendo assim, o uso de uma alternativa adjuvante, como a PBM, que acelere esse processo, aumentando o metabolismo celular, melhorando a regeneração celular e promovendo uma resposta tecidual, sem causar danos celulares, se torna ideal para o tratamento de dentes permanentes necrosados com rizogênese incompleta (ALMEIDA-LOPES et al., 2001; SALATE et al., 2005; TUBY, MALTZ, ORON, 2007; HOU et al., 2008; MVULA et al., 2008; TOOMARIAN et al., 2012).

Atualmente, os estudos têm dado ênfase à utilização da PBM devido a sua influência no metabolismo celular, síntese de colágeno, capacidade de regular a expressão de alguns fatores de crescimento e genes relacionados à diferenciação das células ósseas, promovendo, assim, a reparação tecidual (ALMEIDA-LOPES et al., 2001; SALETE et al., 2005; DE SOUZA et al., 2011; PRETEL, LIZARELLI, RAMALHO, 2007; TIM et al., 2015). Estudos in vitro recentes demonstraram que a PBM aumentou a expressão gênica de marcadores osteoblásticos, como a fosfatase alcalina (ALP), o fator de transcrição 2 (Runx-2), a osteocalcina (CO), o colágeno tipo I (COL I), o colágeno tipo III (COL III), sialoproteína óssea (BSP), factor de crescimento transformante 2 (TGF-β), proteínas morfogenéticas ósseas (BMPs), fator de crescimento de fibroblastos (FGF) (FERREIRA et al., 2006; DA SILVA et al., 2012; ARANY et al., 2014; TIM et al., 2015).

Recentemente, o efeito estimulador da PBM no desenvolvimento de raízes de dentes pulpotomizados proporcionou uma cicatrização tecidual satisfatória (FERNANDES et al., 2015; MARQUES et al., 2015). Alem disso, a PBM acelerou a taxa dentinogênese e apicigênese de dentes hígidos permanentes imaturos (TOOMARIAN et al., 2012; FEKRAZAD et al., 2015). Entretanto, torna-se necessário avaliar o efeito desta terapia em protocolos de tratamento de dentes permanentes com necrose pulpar e rizogênese incompleta.

2. OBJETIVOS

2.1 OBJETIVOS GERAIS

O presente estudo teve como objetivo avaliar, in vitro, a influência da fotobiomodulação (PBM) na regulação epigenética, no potencial de migração e na diferenciação de células tronco de dentes permanentes humanos (hDPSCs) de terceiros molares hígidos; e, in vivo, avaliar a influência da PBM na indução da complementação da formação radicular de molares de ratos com necrose pulpar e rizogênese incompleta.

2.2 OBJETIVOS ESPECÍFICOS

a) Avaliar, in vitro, a influência da PBM sobre a viabilidade celular das hDPSCs, pelo ensaio de MTT;

b) Avaliar, in vitro, a influência da PBM sobre a migração celular das hDPSCs, pelo método de *Scratch*;

c) Avaliar, in vitro, a influência da PBM sobre a atividade epigenética das hDPSCs, através da imunoflurescência do marcador Acetyl histona H3;

d) Avaliar, in vitro, a influência da PBM sobre a capacidade de diferenciação osteogênica, adipogênica e condrogênica das hDPSCs e atividade ALP, em cultura 3D, nos períodos de 7 e 14 dias;

e) Avaliar, in vivo, a influência da PBM na resposta tecidual de molares de ratos com necrose pulpar e rizogênese incompleta, submetidos a diferentes protocolos de tratamento;

f) Imunolocalizar, pelo marcador Sialoproteina Óssea (BSP), a deposição de tecido mineralizado nas paredes e região apical de canais radiculares de molares de ratos com necrose pulpar e rizogênese incompleta, submetidos a diferentes protocolos de tratamento.

3 CAPÍTULO 1

Effect of photobiomodulation therapy on histone acetylation of human dental pulp stem cells.

I. M. Zaccara[1], L. B. Mestieri[1], A. P. Pilar EFS[2], M. S. Moreira[3], F. S. Grecca[1], M. D. Martins[1], P. M. P. Kopper[1]

[1] Department of Conservative Dentistry, School of Dentistry, Federal University of Rio Grande do Sul - UFRGS, Porto Alegre, RS, Brazil.

[2] Department of Experimental Pathology, Clinics Hospital of Porto Alegre, Federal University of Rio Grande do Sul - UFRGS, Porto Alegre, RS, Brazil

[3]Department of Dentistry, School of Dentistry, University of Sao Paulo, Brazil; Ibirapuera University, Sao Paulo, Brazil.

Running Head: PBM therapy improves histone acetylation

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Correspondence author:

Patrícia Maria Poli Kopper

Division of Endodontics, Department of Conservative Dentistry, School of Dentistry, Federal University of Rio Grande do Sul - UFRGS, Porto Alegre, RS, Brazil.

Av. Ramiro Barcelos, 2492

Porto Alegre-RS, Brazil.

CEP: 90035-003

Telephone: 55 51 3308 5191

e-mail: pkopper@terra.com.br

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ABSTRACT

Aim: To evaluate *in vitro* the role of PBM on human dental pulp stem cells (hDPSCs) viability and migration related to histone acetylation.

Methodology: The hDPSCs were characterized and assigned into two groups: Control and PBM. For the PBM group, five laser irradiations at 6h intervals were performed using a continuous wave InGaAlP diode laser (power 100 mW, wavelength 660 nm, energy density of 3J/cm^2, 10s of exposure time totalizing 1J per point). Viability (MTT assay), migration (scratch assay), and acetylation of histone H3 (H3K9ac immunofluorescence) were evaluated immediately after the last laser irradiation (24 hours). Data were analyzed by Mann-Whitney or T test (α=5%).

Results: PBM significantly increased the hDPSCs viability ($P<0.001$) and migration ($P<0.05$). Until the first 12 hours, PBM group significantly accelerated wound closure compared to the control ($P<0.001$) and the wound was closed at 18 hours, being faster than the control, which finished the process at 24h ($P<0.05$). PBM induced epigenetic modifications in hDPSC due to increased acetylation of histones when compared to the control group ($P<0.001$).

Conclusion: PBM increased viability and migration of hDPSCs that are related with the up-regulation of histone acetylation, and could be considered a promising adjutant therapy for regenerative endodontic treatment.

INTRODUCTION

The clinical regenerative strategies in Endodontics involve a triad of tissue engineering (stem cells, biomaterials, inductive growth and differentiation factors) and have been demonstrating potential to replace damaged structures, such as dentin and pulp complex cells. One important strategy is the stimulation of mesenchymal stem cells (MSC) capacity of self-renewal and differentiation into some cell types (Hargreaves *et al.* 2013, Schmalz & Smith 2014, Ginani *et al.* 2015, Marques *et al.* 2016).

In this context, photobiomodulation therapy (PBM) has been shown to promote biostimulatory effects *in vitro* and *in vivo* in different cell types including MSC (Marques *et al.* 2016). PBM is described as a form of light therapy that utilizes non-ionizing forms of light sources, including lasers, LEDs, and broadband light, in the visible and infrared spectrum. It is a nonthermal process involving endogenous chromophores eliciting photophysical (i.e., linear and nonlinear) and photochemical events at various biological scales (Anders *et al.* 2015). PBM could stimulate cell growth, increase cell metabolism, improve cell regeneration, promote tissue response, and accelerate dentin regeneration after pulp exposure (Salate *et al.* 2005, Pretel *et al.* 2007, De Souza *et al.* 2011). A large number of signaling proteins have been enrolled in the process of PBM-induced cell proliferation, migration and differentiation. This therapy activates cellular metabolites in the mitochondrial respiratory chain and activates various metabolic processes by the release of signaling molecules, including reactive oxygen species (ROS), synthesis of DNA, RNA, proteins, enzymes, and other products (Karu *et al.* 1989, Gao & Xing 2009, Passarella & Karu 2014). However, the impact of PBM in epigenetics mechanisms was not described.

Epigenetic mechanisms, also called ‘epigenetic code’, refer to the biological mechanism by which gene expression in the genome can be regulated without altering the base DNA sequence (Wu & Sun 2006, Javaid & Choi 2017). The key processes responsible for epigenetic regulation are DNA methylation, histone modifications and posttranscriptional gene regulation by noncoding RNA, commonly referred as microRNAs (Javaid & Choi 2017). These mechanisms are involved in maintenance of tissue-specific genetic signatures regulating the proliferation and differentiation of different cell types in both prenatal and postnatal development in

response to environmental, developmental and metabolic cues (Arnsdorf *et al.* 2010). Among the epigenetic mechanisms mentioned above, histone modification has been shown to be important for different cell function-regulating gene expression in several tissue types (Javaid &Choi 2017 Kruhlak *et al.* 2001, Boland *et al.* 2014).

Histones are the most abundant proteins associated with DNA, and the cellular behavior depends on how tightly DNA is spooled around H2A, H2B, H3 and H4 core histones. Histone modifications determine chromatin status (euchromatin or heterochromatin), the accessibility of DNA to nuclear factors and ultimately the transcription. Alterations in chromatin structure due to histone modifications have been correlated with gene expression, cell cycle, DNA replication and damage, DNA repair, and chromosome stability (Boland *et al*. 2014, Martins & Castilho 2013). Furthermore, lysine acetylation, serine phosphorylation, lysine methylation, arginine methylation and lysine ubiquitination are the majority of posttranslational modifications in histones (Boland *et al.* 2014; Martins & Castilho 2013). Histone modification by acetylation is able to induce an open chromatin structure, which correlates with gene activation. Conversely, histone deacetylation results in condensation of chromatin, which is related to transcriptional repression (gene silence). The acetylation of histone H3 observed at Lys9, 14, 18, 23, 27, and 56 is associated with gene activity (Thiagalingam *et al.* 2003, Jayani *et al.* 2010). Of interest, functional acetylation of histone H3 at Lys 9 (H3K9ac) controls chromatin decondensation, chromatin assembly, and gene activation, being a great marker of transcriptionally active chromatin (Ferrari & Strubin 2015).

The epigenetic regulation related to regeneration and pulp repair has been studied by evaluating the inhibitors of histone deacetylases (HDACi), which increases genetic transcription (Duncan *et al.* 2012, 2013, 2016; Hui *et al.* 2016). Besides, it has been indicated that epigenetic regulation of histone proteins controls cell phenotype and regulates the renewal and pluripotency of stem cell populations (Duncan *et al.* 2016). The importance of histone acetylation in MSC has been established (Duncan *et al.* 2016); additionally, it is well known that photobiomodulation (PBM) promotes the biomodulation and proliferation in human dental pulp stem cells (hDPSCs) (Eduardo *et al.* 2008, Arany *et al.* 2014, Zaccara *et al.* 2015). The field of epigenetics presents a unique opportunity to understand the molecular mechanisms underlying the modulation of gene expression in the absence

of alterations to the DNA sequence. Considering the absence of evidence about the effect of PBM on histone acetylation of MSC, the present study aimed to evaluate, *in vitro,* the role of PBM on hDPSCs viability and migration related to histone acetylation.

MATERIALS AND METHODS

Subjects

The study was approved by the Institutional Review Board of the Federal University of Rio Grande do Sul, Brazil (CAAE: 45459615.8.0000.5347). Human DPSCs were obtained from four healthy permanent third molars with incomplete root formation indicated for extraction due to orthodontic reasons, from two healthy patients aged 18 years old. All participants signed an informed consent statement prior to any clinical procedure.

Cell culture

After extraction, the apical papilla was removed and the pulp tissue was isolated from apical opening using Hedströem files (Maillefer, Dentsply, Switzerland) and stored in a culture dish (35x10 mm) containing Alpha Modification Minimum Essential Eagle's Medium (α-MEM, Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin and streptomycin (PenStrep, Gibco). The pulp tissues were sectioned into fragments of approximately 1 mm^3 and kept in supplemented α-MEM at 37°C, 95% humidity and 5% CO^2. The culture medium was changed 24h after pulp collection and at every 48h to obtain explants of hDPSCs. After reaching the confluence (70-90% of cells) of the plate, the cells were trypsinized (Trypsin, Sigma-Aldrich) to the next passage.

Stem cell characterization

In the second passage (P2), the hDPSCs were analyzed to confirm their stem cell nature. Briefly, an aliquot of cells was evaluated by flow cytometry, which revealed positive staining for surface markers of mesenchymal stem cells (STRO1, CD146 and CD44) and negative staining for markers of hematopoietic stem cells (CD45 and CD14) (all from Santa Cruz Biotechnology, USA). Additionally, the multilineage differentiation potential of hDPSCs, up to 14 days, was confirmed by culturing the cells in osteogenic, adipogenic and chondrogenic differentiation mediums.

Experimental protocol

Human DPSCs from the third passage (P3) were plated and maintained in culture medium in a humified chamber at 37ºC. Twenty hours later, the PBM group was submitted to the first irradiation protocol. The control group was not irradiated. All cell culture experiments were performed under laminar flow (biosafety class II), and cells were monitored daily using a phase contrast microscope (Axio Observer Z1 microscope, Zeiss, Göttingen, Germany).

Photobiomodulation Therapy

Laser irradiation was applied with a continuous-wave indium-gallium-aluminum-phosphide (InGaAlP) diode laser (MM Optics Ltd., São Carlos, SP, Brazil) using the following parameters: wavelength of 660 nm, power of 100 mW, spot size of 0.3cm^2, energy density of 3J/cm^2, 10 seconds of exposure time and 1J of energy per point of application. Five laser applications were made at 6-hour intervals (Meneguzzo *et al.* 2010), resulting in a total energy of 5J in each point. The laser probe was fixed perpendicular to each tissue-culture plate. Since the distance between the laser source and the surface of application is critical, the PBM was administered through the bottom of the optically clear plates. The cells were plated in a way to maintain empty wells between seeded wells, in order to minimize the unintentional dispersion of light between wells during laser application. The output

power of the equipment was tested using a power meter (Laser Check; MM Optics LTDA, Sao Paulo, Brazil) before and after irradiations.

Cell viability assay

For cell viability, $1x10^4$ of hDPSCs were cultured in culture medium αMEM supplemented with 1% penicillin and streptomycin and regular 10% FBS were seeded in 96-wells plate (Friedrich, 2009). After 24 hours of hDPSCs adhesion, the culture medium was replaced and, in the PBM group, laser irradiations were performed according to the previously described parameters.

Cell viability was assessed by 3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-tetrazolium bromide (evaluation of mitochondrial activity by reduction of MTT), immediately after the last laser irradiation. Cells were incubated with MTT (Sigma-Aldrich) at 37°C for 3h, following the manufacturer's protocol. After this period, the well contents were removed and colorimetric product was solubilized in 100 µL of 0.04 N acidified isopropanol (Sigma-Aldrich). The optical densities of the solutions were measured in a spectrophotometer (Thermo Fischer Scientific Inc, Waltham, MA, USA) at 570 nm wavelengths. The absorbance readouts were normalized with the absorbance of the control group and represented the cell viability percentage.

Scratch-wound migration assay

To assess the migratory ability of hDPSCs upon PBM therapy, an *in vitro* scratch-induced wound model was used. Human DPSCs cells ($1x10^6$) were seeded on 35mm culture plates and maintained at 37°C to confluence in culture medium. After 24 hours of hDPSCs adhesion, a wound was made in the monolayer of cells by completely scratching the cells in a line with a 200-µl pipette tip. The first irradiation was performed immediately after scratching (T0). A total of five irradiations were applied in four points with 6-h intervals between each application. Cells were allowed to proliferate and migrate into the scratch wound for 24h since T0. Migration of cells into the wound was photographed at 0, 6, 12, 18 and 24h under a phase contrast microscope (Axio Observer Z1 microscope, Zeiss, Göttingen, Germany), using a coupled camera (Axiocam mrn, Zeiss) with 10x magnifying lens (Ecplan-NEOFLUAR

10x / 0.3 aperture, Zeiss). The assay was performed in replicate with analysis of 8 areas in each group. Relative wound closure (%) was measured using a computerized image analyzer system (Image J software).

Immunofluorescence of histones H3 acetylation

Histone acetylation was assessed by immunofluorescence of acetyl histone H3. Human DPSCs were placed on glass coverslips inside 6-well plates and maintained at 37°C in culture medium (3 wells per group). After 24 hours of hDPSCs adhesion, the culture medium was replaced and, in the PBM group, laser irradiations were performed in four points on each well, according to the previously described parameters. The first irradiation was applied immediately after cell adhesion, and then more four irradiations were performed with 6-h intervals between each application. After that, the immunofluorescences were performed. The cells were fixed with absolute methanol at -20°C for 5 min. Cells were blocked with 0.5% (v/v) Triton X-100 in PBS and 3% (w/v) bovine serum albumin and incubated with anti-acetyl-histone H3 at lys9 (H3K9ac, 1:100, Cell Signaling Technology, Danvers, Massachusetts). Cells were then washed three times, incubated with TRITC-conjugated secondary antibodies (ab6718, Abcam, Eugene, Oregon, USA) diluted to 1:500 in the dark for 90 minutes, and stained with DAPI (ab104139 – Abca, Eugene, Oregon, USA) for 5 minutes for visualization of DNA content. Analysis of acetyl-histone H3 immunofluorescence was performed using confocal laser scanning microscopy (Leica TCS SP8, Leica Microsystems, Wetzlar, Germany) at 400x magnification. A total of 12 images of each group were obtained using the LAS X Software (Leica Microsystems, Wetzlar, Germany). A total of 100 cells were counted using the software Image J (Version 1.38s; NIH, Bethesda, MD), and then the percentage of marked cells, related to DAPI stained cells, were calculated.

Statistical analysis

The Mann-Whitney test was employed for comparison of cell viability and scratch data (nonparametric data). The T test compared data from acetylation of histones H3 assay (parametric data). Data were analyzed using GraphPad Prism 5

(GraphPad Software Inc., San Diego, CA, USA) and the significance level was set at $P<0.05$.

RESULTS

Figure 1 summarizes comparison between PBM and control groups in viability, scratch and immunofluorescence assays. Figures 2 and 3 illustrate the migration and histones H3 acetylation, respectively.

PBM significantly increased hDPSC cell viability when compared to control group ($P<0.001$) (figure 1A). PBM accelerated the healing by activation of hDPSCs migration ($P<0.05$). Until the first 12 hours, PBM group showed significantly increased migration of cells in the wound compared to the control ($P<0.001$). At 18 hours, PBM group promoted the closure of the wound, being faster than the control, which only completely closed the wound at 24h ($P<0.05$) (Figure 1B). PBM induced epigenetic modifications in hDPSC due to increased acetylation of histones when compared to the control group ($P<0.001$) (Figure 1C).

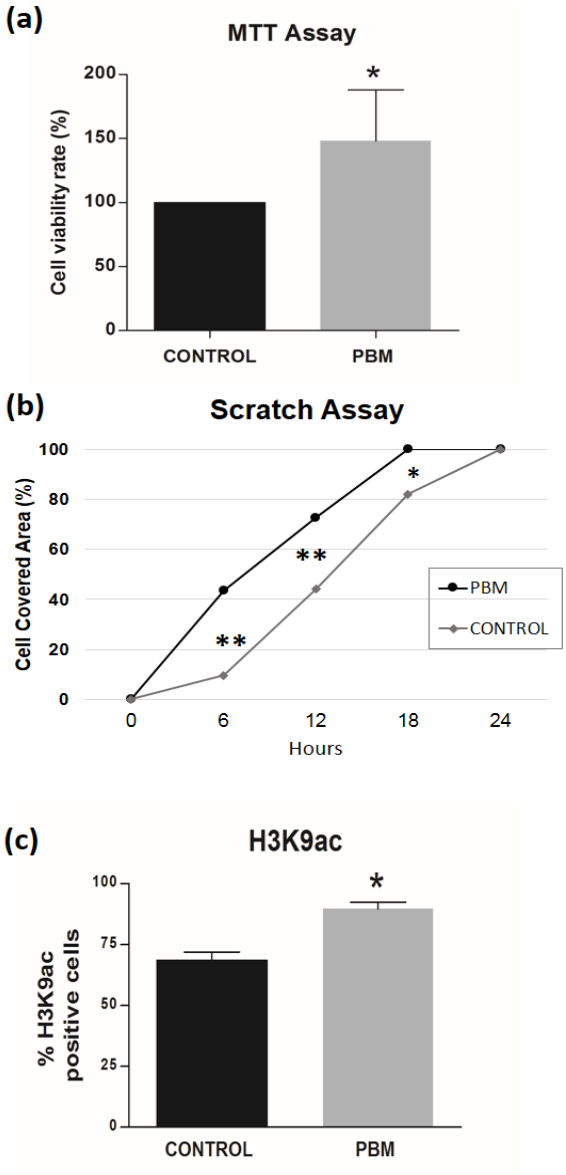

Figure 1: Graphic representation of comparison between PBM and control groups. (A) MTT assay, expressed as percentage of cell viability (%). Asterisk represents significant difference between groups (P<0.001). (B) Scratch assay, expressed as percentage of cell-covered area, during 24 hours. One (P<0.05) and two asterisks (P<0.001) indicate significant difference between groups. (C) Immunofluorescence assay, expressed as percentage of acetyl-histone H3 (H3K9ac) positive cells. Asterisk represents significant differences between groups (P<0.001).

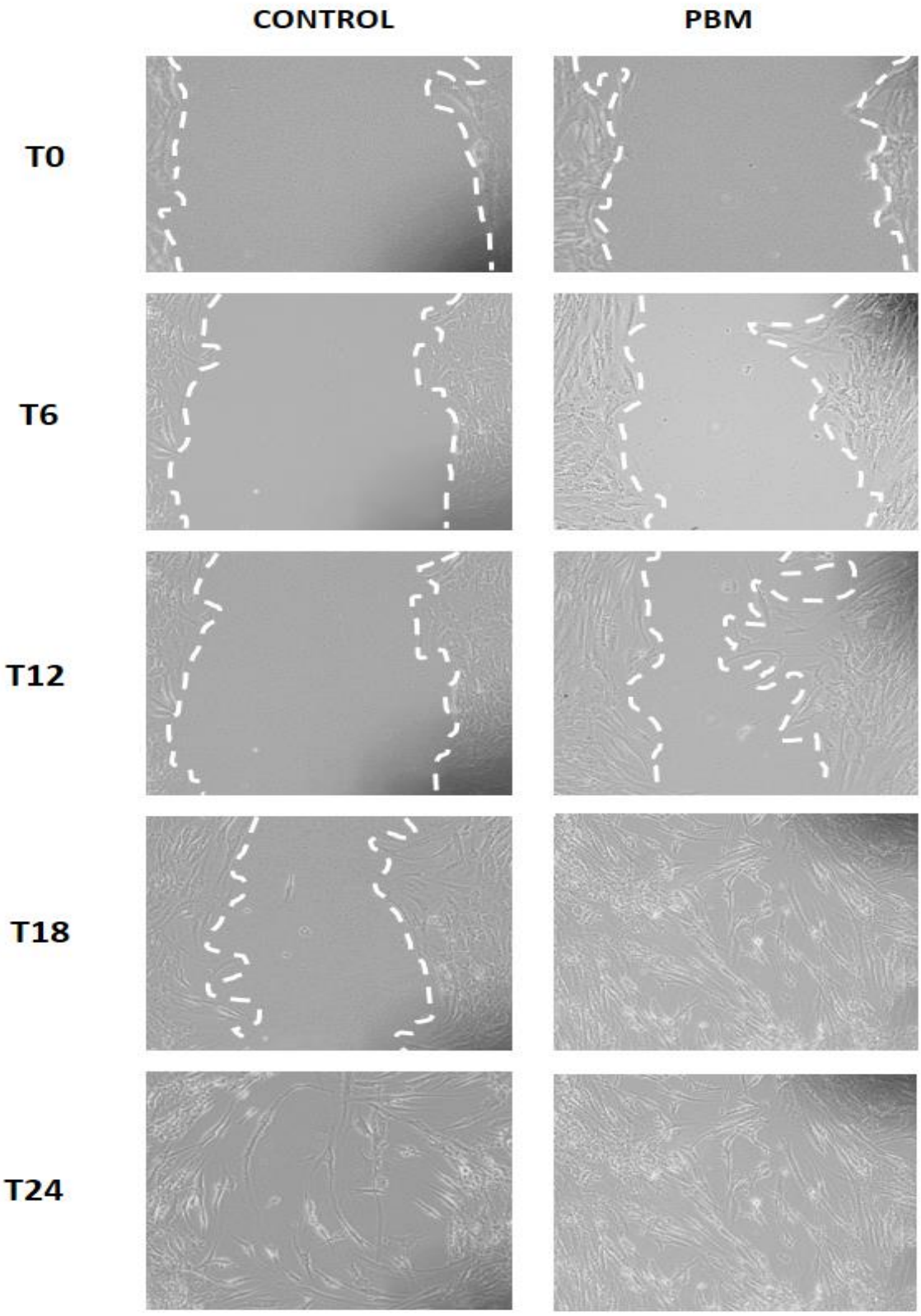

Figure 2: Images of the migration of hDPSCs (scratch assay), captured on Axio Observer Z1 microscope (100x magnification), in the PBM and control groups, during the entire experimental time: T0- wounds generated after cell confluence; T6- wounds 6 hours after cell confluence; T12- wounds 12 hours after cell confluence; T18- wounds 18 hours after cell confluence; T24- wounds 24 hours after cell confluence.

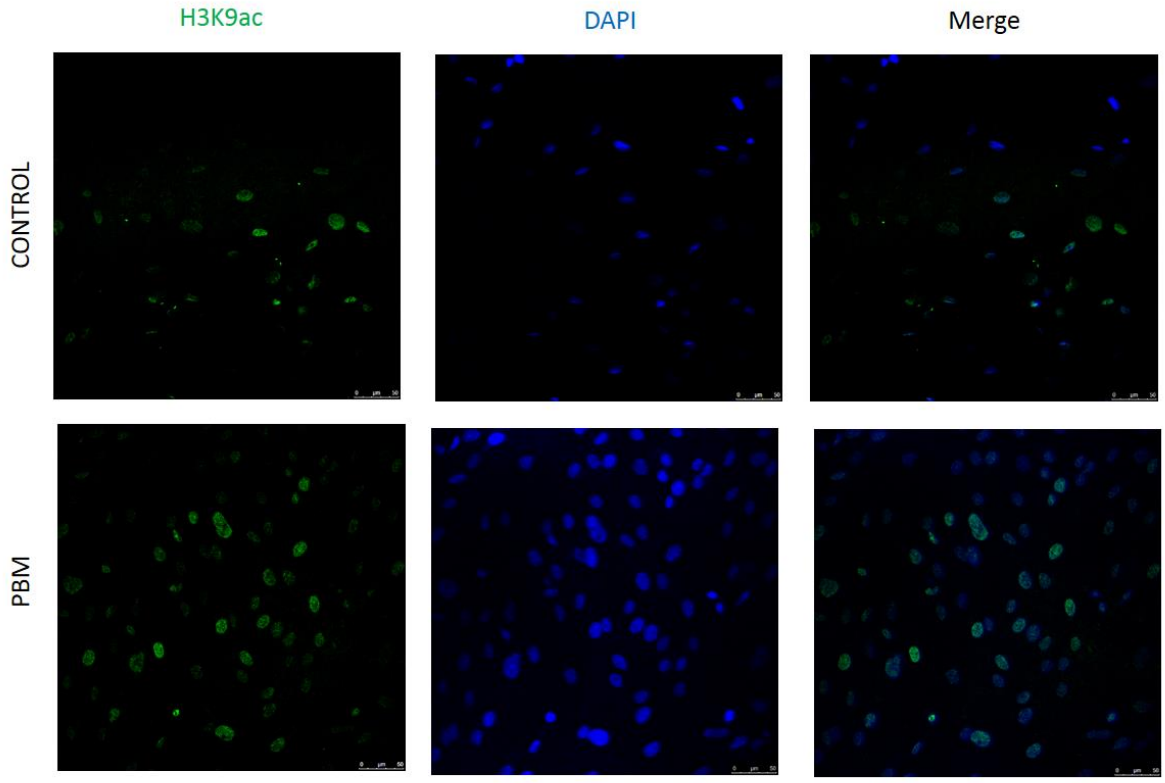

Figure 3: Images of acetylation of histones H3 in hDPSCs at PBM and control groups, captured in confocal laser scanning microscopy (400x magnification). H3K9ac - cells stained by histone H3; DAPI - cells stained by DAPI; Merge - superposition of H3K9ac and DAPI stained cells.

DISCUSSION

The properties of self-renewal and the multilineage differentiation potential into functional odontoblasts and vascular endothelial cells presented by hDPSCs suggest that they can serve as a source for studies in dental pulp tissue engineering and physiological regeneration of the dentin-pulp complex (Piva *et al.* 2014, Schmalz *et al.* 2014). Identifying factors that could promote proliferation and differentiation of hDPSCs is outstanding. Also, understanding the mechanisms used for cellular regulation is important to devise novel treatment strategies (Duncan *et al.* 2016). Based on that, the present study evaluated the impact of PBM in hDPSCs viability, migration and epigenetic mechanisms. The present results revealed that laser irradiation with diode increased hDPSCs viability, stimulating the migratory capacity of this cell type. All these modifications were related to the increase of H3K9ac labeling in hDPSCs, indicating chromatin acetylation.

An abundant quantity of cells is necessary for regenerative therapy, that could be reached more quickly with the aid of PBM, which has been shown to induce stem cell activity (De Villiers *et al.* 2011). Until now, there is no specific parameter to employ the PBM therapy, yet there is an interval of parameters that present positive results of cell viability, migration, proliferation and differentiation, summarized by some systematic reviews (Ginani *et al.* 2015, Borzabadi-Farahani 2016, Marques *et al.* 2016). Furthermore, irradiation could influence the cellular metabolism when visible light spectrum (600 to 700 nm) is used and when the energy density ranges from 0.5 to 4 J/cm^2. However, an energy density higher than 10 J/cm^2 could damage photoreceptors, reducing the biomodulation effect (Emelyanov & Kiryanova 2015). In accordance with the current findings, the literature has shown that the use of PBM improves the results of cell migration (Eduardo *et al.* 2008, Horvát-Karajz *et al.* 2009, Soares *et al.* 2013, Zaccara *et al.* 2015).

The effects of PBM therapy on stem cells have been mostly investigated by proliferation and viability assays (Marques *et al.* 2016). Considering that PBM excites molecules of the mitochondrial respiratory chain, intensifying the formation of a transmembrane electromechanical proton gradient in mitochondria, increasing cell viability due to the large release of calcium into the cytoplasm, which triggers mitosis (Karu 1989, Friedmann *et al.* 1991), it is important to investigate cell viability by the MTT assay. Herein it was demonstrated that PBM increased the viability of hDPSCs, and these findings are in agreement with some previous studies (Eduardo *et al.* 2008, Soares et al. 2013, Zaccara et al. 2015, Moura-Netto *et al.* 2016). Moreover, several studies have demonstrated that PBM has a dose-dependent effect on biological responses according to the number of applications (Huang *et al.* 2009, Soares *et al.* 2013, Zaccara *et al.* 2015,). The best effect of PBM was observed with intervals of 6 hours of irradiation (Meneguzzo *et al.* 2010). However it is necessary to validate its results with other viability or cell migration assay, such as scratch (wound healing model).

Thus, the scratch assay was employed to evaluate the PBM effect on hDPSCs during cell migration. In this experimental model, an artificial gap is created on a confluent cell monolayer culture and the cell migration rate is observed until the gap closes (Liang *et al.* 2007). The present results demonstrated that PBM significantly influenced the migration, validating the data of the MTT assay. These

findings are in agreement with some previous in vitro investigations (Tschon *et al.* 2015, Gagnon *et al.* 2016). According to them, PBM plays an important role in the improvement of wound healing and increases cellular migration when used with an energy density until 10 J/cm^2 (Tschon *et al.* 2015, Gagnon *et al.* 2016). Arany *et al.* (2014) observed that treatment with PBM with multiple irradiations induces reactive oxygen species (ROS) and activates the endogenous latent transforming growth factor-b1 (LTGF-b1), which directs the stem cell for proliferation and differentiation. In addition, the ROS can directly activate the nuclear factor kappa B (NFkB), which is a transcription factor that regulates the expression of various genes related to many cellular functions. The increased NF-κB production after PBM irradiation enhances gene transcription that leads to reduced cell death, cell proliferation and migration (De Freitas & Hamblin, 2016).

Moreover the chromophore cytochrome c oxidase (Cox, unit IV in the mitochondrial respiratory chain), which acts as a photoacceptor and transducer of photosignals in the red and near-infrared regions of the light spectrum, increases an electron transport, mitochondrial membrane potential and ATP production. It occurs due to photons dissociate inhibitory nitric oxide from the Cox. After the initial photon absorption events, numerous signaling pathways are activated via reactive oxygen species, cyclic adenosine monophosphate (cAMP), nitric oxide (NO) and Ca2+, leading to activation of transcription factors. These transcription factors can lead to an increased expression of genes related to protein synthesis, cell migration and proliferation, anti-inflammatory signaling, anti-apoptotic proteins, antioxidant enzymes (De Freitas & Hamblin, 2016). These molecular processes are fundamental for wound healing or regeneration of cellular components (Arany *et al.* 2014).

To our knowledge, the impact of PBM in epigenetics mechanisms in mesenchymal stem cells was not yet described. In this investigation, the effect of PBM in acetylation of histone H3 of hDPSCs, using the same parameters and time of analysis (24 hours) of viability and migration assays, was evaluated. Thus, the results of acetylation and proliferative potential could be correlated. Epigenetic regulation is involved in the maintenance of tissue-specific genetic signatures regulating the proliferation and differentiation of different cells types (Arnsdorf *et al.* 2010). Among the epigenetic mechanisms, a histone modification has been shown to be important for different cellular function regulating gene expression in several tissue types

(Kruhlak *et al.* 2001, Boland *et al.* 2014, Javaid & Choi 2017). In this way, it is determined that histone modification is an essential epigenetic mechanism, since it can alter chromatin states either by acetylation or methylation (Seo *et al.* 2015). Histone acetylation represents the addition of acetyl groups, by histone acetyl transferases (HAT) enzymes, to the lysine residues located in the histone tails (Zupkovitz *et al.* 2006). This mechanism neutralizes these lysine residues, weakens the interaction of histone tails with the local DNA, negatively loaded, inducing local opening and decompressing the chromatin structures (Zupkovitz *et al.* 2006). In contrast, the histone deacetylase (HDAC) enzyme induces chromatin compression (Zupkovitz *et al.* 2006).

Currently, studies have shown that epigenetics regulates hDPSCs differentiation status and self-renewal, by modulating the balance between HDAC and HATs of these cells (Seo *et al*. 2015, Duncan *et al.* 2016). It demonstrates an encouraging endodontic perspective for regenerative therapy in immature necrotic permanent teeth (Paino *et al.* 2014). Therefore, it becomes interesting to identify mechanisms with potential to up-regulate the epigenetic events (Duncan *et al*. 2012). Some studies have described several types of histone deacetylase inhibitors (HDACi), including trichostatin A (TSA), valproic acid (VPA) and butyric acid, which reversibly alter histone acetylation, promoting gene transcription and then inducing proliferation, differentiation and anti-inflammatory effects in MSCs (Jin *et al.* 2013, Mahmud *et al.* 2014, Duncan *et al.* 2016, Luo *et al.* 2017). Luo *et al.* (2017) observed that HDACi promoted proliferation, migration and adhesion of hDPSCs, suggesting that epigenetic regulation may contribute to novel regenerative therapies for pulp disease treatment. Moreover, histone acetylation in the residue lysine 9 of histone H3, evaluated in the present study, is the most common modification associated with active transcription and increase of gene expression (Thiagaligam *et al.* 2003). Thus, the acetylation of residue lysine 9 of histone H3 has been considered an epigenetic marker, representing a decompressed chromatin and permitting the binding of transcription factors (Saraiva *et al.* 2010, Martins & Castilho 2013).

Therefore, to our knowledge, the effect of PBM on epigenetic regulation of hDPSCs has not been evaluated so far, specifically on Acetyl-Histone H3 (Lys9) expression. The present investigation demonstrated that PBM can modulate histone acetylation signaling of hDPSCs, thus it could be inferred that PBM plays a similar

role to HDACi, promoting nuclear modifications that chemically induce histone acetylation, generating gene expression to increase the cell viability and migration.

CONCLUSION

The results of the present investigation showed that PBM increased viability and migration of hDPSCs that are related with the up-regulation of histone acetylation. Thus, PBM could be considered a promising adjutant therapy for regenerative endodontic treatment.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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4 CAPÍTULO 2

Photobiomodulation improves multi-lineage differentiation of dental pulp stem cells in an 3D culture model

Ivana M. Zaccara [1], Letícia B. Mestieri[1], Maria Stella Moreira[2], Fabiana S. Grecca[3], Manoela D. Martins[4], Patrícia M. P. Kopper[3]

[1] MSc, Department of Conservative Dentistry, School of Dentistry, Federal University of Rio Grande do Sul - UFRGS, Porto Alegre, RS, Brazil.
[2] PhD, Department of Dentistry, School of Dentistry, University of Sao Paulo, Brazil; Ibirapuera University, Sao Paulo, Brazil.
[3] PhD, Professor of Endodontics, Department of Conservative Dentistry, School of Dentistry, Federal University of Rio Grande do Sul - UFRGS, Porto Alegre, RS, Brazil.
[4] PhD, Professor of Pathology, Department of Conservative Dentistry, School of Dentistry, Federal University of Rio Grande do Sul - UFRGS, Porto Alegre, RS, Brazil.

Author Contact Information:

Ivana M. Zaccara, MSc. Tel: +55 83 993271533. Email: ivana_zac@hotmail.com

Letícia B. Mestieri, MSc. Tel: +55 51 981361708. Email: leticiabmestieri@gmail.com

Maria Stella Moreira, PhD. Tel: +5511 3091-7839. Email: sstella2005@yahoo.com.br

Fabiana S. Grecca, PhD. Tel: +55 51 33085191. Email: fabiana.grecca@ufrgs.br

Manoela D. Martins, PhD. Tel: +55 51 33085011. Email: manomartins@gmail.com

Correspondence author:

Patrícia Maria Poli Kopper

Av. Ramiro Barcelos, 2492, Santa Cecília

Porto Alegre-RS, Brazil.

CEP: 90035-003

Telephone: +55 51 3308 5191

e-mail: patricia.kopper@ufrgs.br

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ABSTRACT

Photobiomodulation (PBM) has shown positive effects on stem cell differentiation in monolayer cell culture model, but little is known about its effect on three-dimensional (3D) agarose gel culture. This study evaluated the capacity of human dental pulp stem cells (hDPSCs) to differentiate into three lineages using 3D agarose culture model, and also to observe the effect of PBM on differentiation potentials under different nutritional conditions. The hDPSCs were characterized and seeded on a 0.3% agarose gel layer with different media (osteogenic, adipogenic, chondrogenic) and were assigned into four groups: Control 10% FBS, Control 5% FBS, PBM 10% FBS and PBM 5% FBS. Irradiation was performed with an indium-gallium-aluminum-phosphide (InGaAlP) laser, 660 nm, with output power of 100 mW, spot size 0.04 cm2, energy density 3J/cm2, 10s of exposure time and 1J of energy per point. The irradiation sessions were applied with 6-h intervals. The irradiated and non-irradiated groups were evaluated at 7 and 14 days. Moreover, ALP activity assay was performed to analyze the deposition of mineralized tissue. Data were quantified and statistically analyzed by Kruskal-Wallis and Dunn tests (α=5%). The hDPSCs cultured in 3D after 14 days exhibited osteogenic, adipogenic and chondrogenic differentiation. Furthermore, PBM demonstrated positive effects significantly stimulating both osteogenic, adipogenic and chondrogenic differentiation processes after 14 days compared to control groups ($p<0.05$). Considering that PBM increased the bioactivity in this experimental model that mimics the natural tissue architecture, it can be concluded that 3D agarose gel culture could be a promising scaffold in tissue engineering applications.

Keywords: Dental pulp stem cells. Low-Level Laser Irradiation. Photobiomodulation. Bioactivity. Differentiation.

INTRODUCTION

Mesenchymal stem cells (MSCs) present important functions such as capacity of self-renewal and development of tissues compatible with their origin (1). The interest in the isolation of human dental pulp stem cells (hDPSCs) has increased substantially in recent years and the teeth has being considered a relatively rich source of MSCs (2). There are many markers to identify MSCs, including the STRO-1 surface marker, which is considered the main positive marker for MSCs and has the characteristic of not marking hematopoietic cells; CD44, a cell adhesion glycoprotein typical of mesenchymal cells; and CD146, a cell adhesion molecule (3). However, these antibodies individually could be insufficient to identify mesenchymal stem cells because of potential cross-reactivity with other cell types (4). Nevertheless, several studies showed that hDPSCs could be characterized by a combination of these three markers together (1, 3, 4). In addition, it is also recommended the analysis of differentiation in multilineage cells to confirm the stemness(5).

The key to use stem cells is their ability to differentiate into various cell types, according to the stimulus received. MSCs present the potential of differentiation into osteoblasts, chondrocytes, adipocytes, muscle and neural tissues according to the microenvironment in which they are located (1). Additionally, photobiomodulation (PBM) has been previously investigated and has been shown to promote biomodulation and increase the proliferation and differentiation of hDPSCs when applied in monolayer culture, representing a beneficial impact on regenerative treatments, such as treatment of dental trauma or necrotic pulp with incomplete apex and periapical lesion, being used as a coadjutant treatment in protocols of repair and root development, accelerating these processes (6, 7, 8, 9).

Two systematic reviews demonstrated that irradiation should influence the differentiation effect when the energy density ranges from 0.5 to 4 J/cm^2 at wavelengths from 600 to 980 nm, and that an energy density higher than 10 J/cm^2 could damage photoreceptors, reducing the biomodulation effect of laser (10, 11). Moreover, according to these systematics reviews, the greatest values of proliferation or differentiation were obtained using high power density, low energy density, and short exposure time (10, 11). Other studies verified a tendency of PBM to improve MSCs viability without deleterious effects; however, considering their method heterogeneity and the limited evidence, it is difficult to obtain a clear conclusion (12).

Despite the important knowledge obtained from studies using monolayer cell culture, they do not accurately recreate the natural tissue architecture (13) and some cell functions can be inhibited, thereby limiting their clinical application and relevance (14). Three-dimensional (3D) cell culture analysis, which can better simulate *in vivo* the cellular conditions, can be a more physiologically relevant and predictive model, presenting greater stability and longer life expectancy than cells in monolayer culture (14). Moreover, the morphology of cells and intercellular signaling are more physiological in 3D cultures than in monolayer culture, thereby promoting better knowledge about cellular function and differentiation *in vivo* (15). Thus, 3D culture analysis seems to be more suitable for viability, cytocompatibility and bioactivity assays (9, 14). Additionally, in 3D culture, when the PBM reaches biological tissues during irradiation, the laser light can be reflected, scattered, absorbed, or transmitted to the surrounding tissues (16). Furthermore, these interactions are also influenced by several specific laser characteristics such as power output, oscillation mode (continuous wave or pulsed wave), or application mode (contact or noncontact).

Among various 3D culture models, the agarose gel (a polysaccharide of D-galactose and 3,6-anhydro-L-galactopyranose derived from the cell walls of red algae) presents favorable characteristics in relation to implantation: it is bioinert, non-toxic, and decreases the potential immune rejection in rats (17). Another important aspect is the positive influence of cultures under nutritional deficit conditions in the response of hDPSCs to PBM, improving cell growth (6). That deficit condition alters the redox state towards oxidation of cells, and the cellular response to PBM is near optimal and stronger when potential redox is shifted (18). Therefore, the present study evaluated the PBM effect on osteogenic, adipogenic and chondrogenic differentiation and phosphatase alkaline activity (ALP) of hDPSCs using an agarose gel 3D model, under different nutritional conditions.

MATERIALS AND METHODS

Subjects

The study was approved by the Institutional Review Board of the Federal University of Rio Grande do Sul, Brazil (CAAE 4545961 5.8.0000.5347). Human DPSCs were obtained from four permanent third molars with incomplete root formation, indicated for extraction due to orthodontic reasons, from two healthy patients aged 18 years old.

Stem cell culture

Freshly extracted teeth were immersed in Alpha Modification Minimum Essential Eagle's Medium (α-MEM, Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Life Technologies, USA) and 1% penicillin and streptomycin (PenStrep, Gibco), and then transported to the laboratory for processing. After extraction, the apical papilla was removed and the pulp tissue was isolated from apical opening using Hedstrom files (Maillefer, Switzerland). After pulp removal, it was sectioned into fragments of approximately 1 mm^3 and kept in supplemented α-MEM at 37°C, 95% humidity and 5% CO^2. The hDPSCs were obtained by explant culture in dishes (35x10 mm) containing α-MEM. The culture medium was changed 24 h after pulp collection and at every 48h to obtain of human dental pulp stem cells (hDPSCs). When reaching the confluence (70-90% of cells) of the plate, the cells were trypsinized (Trypsin,Sigma-Aldrich) to the next passage.

Stem cell characterization

In the second passage (P2), the hDPSCs were analyzed to confirm their stem cell nature. Briefly, an aliquot of cells was evaluated by flow cytometry, which revealed positive staining for surface markers of mesenchymal stem cells (STRO1, CD146 and CD44) and negative staining for markers of hematopoietic stem cells (CD45 and CD14) (all from Santa Cruz Biotechnology, USA). Additionally, the multilineage differentiation potential of hDPSCs, up to 14 days, was confirmed by culturing the cells in varied differentiation media:

a- osteogenic differentiation: αMEM supplemented with 1% penicillin and streptomycin, 10% FBS, 100 nM dexamethasone (Sigma-Aldrich, USA), 0.05 µM ascorbate-2-phosphate (Sigma-Aldrich, USA) and 10 mM β-glycerophosphate (Sigma-Aldrich, USA) (19);

b- adipogenic differentiation: αMEM supplemented with 1% penicillin, 10% FBS, 1 mmol/L dexamethasone, 5 µg/mL^{-1} bovine insulin (Sigma-Aldrich, USA), 0.5 mmol/L iso-butyl-methyl-xanthine (Sigma-Aldrich, USA), and 60 mmol/L indomethacin (Sigma-Aldrich, USA) (20);

c- chondrogenic differentiation: αMEM supplemented with 1% penicillin and streptomycin, 10% FBS, 50 nmol/L ascorbic acid 2-phosphate, 6.25 mg/mL bovine insulin, and 10 ng/mL transforming growth factor–beta 1 (TGF-b1/ Sigma-Aldrich, USA) (21).

Three-dimensional (3D) culture and hDPSC differentiation

A pilot study was conducted to confirm the possibility of analysis of osteogenic, adipogenic and chondrogenic differentiation of hDPSC in three-dimensional (3D) culture. To obtain the 3D culture, a 0.3% agarose solution (Sigma-Aldrich, St. Louis, MO, USA) was prepared in αMEM and 100 uL of this solution were pipetted into each well of a 96-well plate (TPP, Trasadingen, Switzerland). After agarose solidification, 1x10^4 of hDPSCs (third passage) cultured in 150 uL of αMEM supplemented with 1% penicillin and streptomycin and regular 10% FBS were seeded in each well. During 14 days, the cells were maintained at 37°C, 95% humidity and 5% CO^2 in osteogenic, adipogenic and chondrogenic differentiation media, and after fixation they were stained with Alizarin Red, Oil Red and Alcian Blue, respectively. Homogeneous cell embedding in agarose was confirmed by light microscopy (100x) and absorbance in spectrophotometry.

Osteogenic, Adipogenic and Chondrogenic Differentiation in 3D culture

Table 1 shows the distribution of all groups according to PBM and nutritional status: regular (10% FBS) or deficit (5% FBS). The 3D culture preparation and seeding of hDPSCs were performed as previously described in the pilot study. After 24 hours of cell adhesion, the cells were induced to differentiation. For that

purpose, they were maintained in osteogenic, adipogenic or chondrogenic media during 14 days. The medium was changed at every 48 hours.

Table 1: Experimental groups according to the photobiomodulation (PBM) and nutritional condition (regular: 10% FBS or deficit: 5% FBS) for each differentiation analysis (osteogenic, adipogenic and chondrogenic).

Groups	PBM	Nutritional Condition
CONTROL 10%	-	Regular
CONTROL 5%	-	Deficit
PBM 10%	+	Regular
PBM 5%	+	Deficit

Photobiomodulation

Laser phototherapy was applied with a continuous-wave indium-gallium-aluminum-phosphide (InGaAlP) diode laser (MM Optics Ltd., São Carlos, Brazil) using the following parameters: wavelength of 660 nm, power of 100 mW, spot size of 0.3cm^2, energy density of 3J/cm2, 10 seconds of exposure time, 1J of energy per point of application, 6-hour intervals (22) during 7 and 14 days consecutively, totalizing 56J of energy in each point.

The laser probe was fixed perpendicular to each plate and in contact with the tissue culture plates. The cells were plated keeping empty wells between seeded wells, to minimize the unintentional dispersion of light between wells during laser application, and the output power of the equipment was tested using a power meter (Laser Check; MM Optics LTDA, Sao Paulo, Brazil). The control group was treated under identical conditions, but with the laser device switched off.

Alizarin Red Staining

To evaluate the cells for their mineralized matrix synthesis potential, after 7 and 14 days the cells were fixed in 10% formaldehyde solution (Sigma-Aldrich) and stained with 2% Alizarin Red (Sigma-Aldrich) (19). Quantitative analysis of the nodules was performed, as proposed by Hessle et al. in 2002 (23). For this, 1.0 mL of cetylpyridinium chloride (Sigma-Aldrich) was added to each well for solubilization of nodules, followed by quantification in an ELISA reader (Thermo Fischer Scientific

in, Waltham, MA, USA) at 570-nm wavelength. The measuring absorbance of a well containing only agarose was subtracted from the absorbance of test groups for the three differentiations below.

Oil Red O Staining

After culture in adipogenic medium, as previously described, staining with Oil Red O dye (Sigma-Aldrich) was performed to visualize lipid vesicles in the cells after 7 and 14 days of culture. The cells were fixed with 2% paraformaldehyde (Sigma-Aldrich) for 30 minutes, washed with PBS and stained with the dye diluted in PBS (6:4) for 1 hour. After this, the cells were washed with PBS and observed under a light microscope (28). Stained Oil Red O was also eluted with 4% Nonidet P-40 (Sigma-Aldrich) in isopropanol (v/v) for further quantification by spectrophotometry at 490nm (24).

Alcian Blue Staining

To evaluate the chondrogenic potential of cells, Alcian Blue (Sigma-Aldrich) dye staining was performed in the cultures after 7 and 14 days. The dye was prepared using 1g of Alcian blue dissolved in 100 mL of 3% acetic acid (Sigma-Aldrich). The cells were fixed with 2% paraformaldehyde (Sigma-Aldrich) for 30 minutes, washed with PBS and stained with the diluted dye in PBS (6:4) for 1 hour (29). Stained Alcian Blue was also eluted with 4% Nonidet P-40 (Sigma-Aldrich) in isopropanol (v/v) for further quantification by spectrophotometry at 490nm (24).

Alkaline phosphatase activity (ALP) in 3D culture

ALP enzyme was used as a marker of osteoblastic activity (Labtest Diagnostica, Lagoa Santa, MG, Brazil) and was measured in hDPSCs cultured in 3D agarose after 7 and 14 days in all groups described in Table 1. The cells were lysed with TRIS 1% (Sigma-Aldrich) for 50 minutes at room temperature and the experiment was performed according to the manufacturer's instructions. The absorbance was measured in triplicate by 96-well plate (TPP) spectrophotometry at

590 nm in a spectrophotometer, the results were calculated and data were expressed as ALP enzyme activity (U/L).

Statistical analysis

All experiments were performed in quadruplicate. Differences between test and control groups were analyzed by Kruskal-Wallis followed by Dunn tests on the software GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA), considering a significance level of 5% ($P<0.05$).

RESULTS

hDPSCs characterization

The cells collected from dental pulp showed positive staining for surface markers of mesenchymal stem cells (STRO1, CD146 and CD44) and negative staining for markers of hematopoietic stem cells (CD45 and CD14) (Figure 1). Moreover, those cells cultured in monolayer during 14 days showed deposition of mineralized matrix as stained by Alizarin Red, lipid vesicles stained by Oil Red O and glycosaminoglycans deposits stained by Alcian Blue, indicating, respectively, the osteoblastic, adipose and chondroblastic differentiation (Figure 2). Thus, according to these results, the cells analyzed could be defined as dental pulp stem cells (hDPSCs).

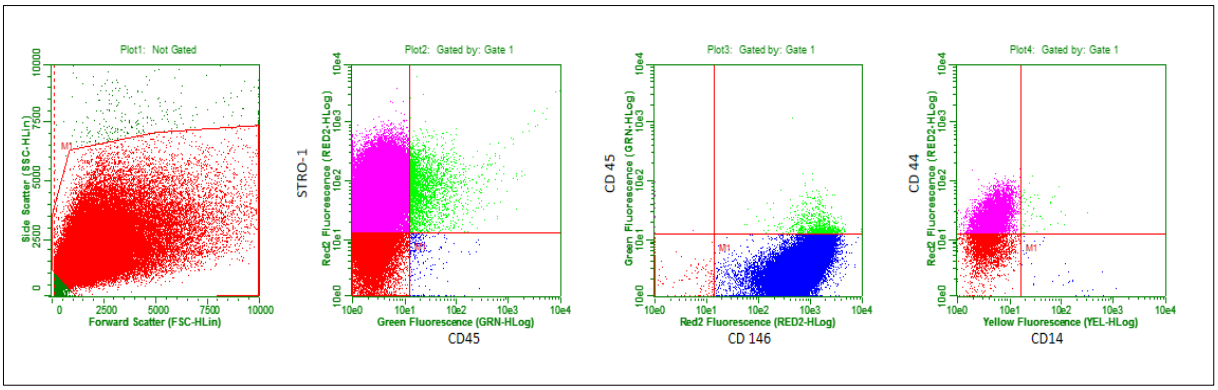

Figure 1: Immunophenotyping of dental pulp stem cells by flow cytometry after two passages. Expression of STRO1, CD146 and CD44 markers.

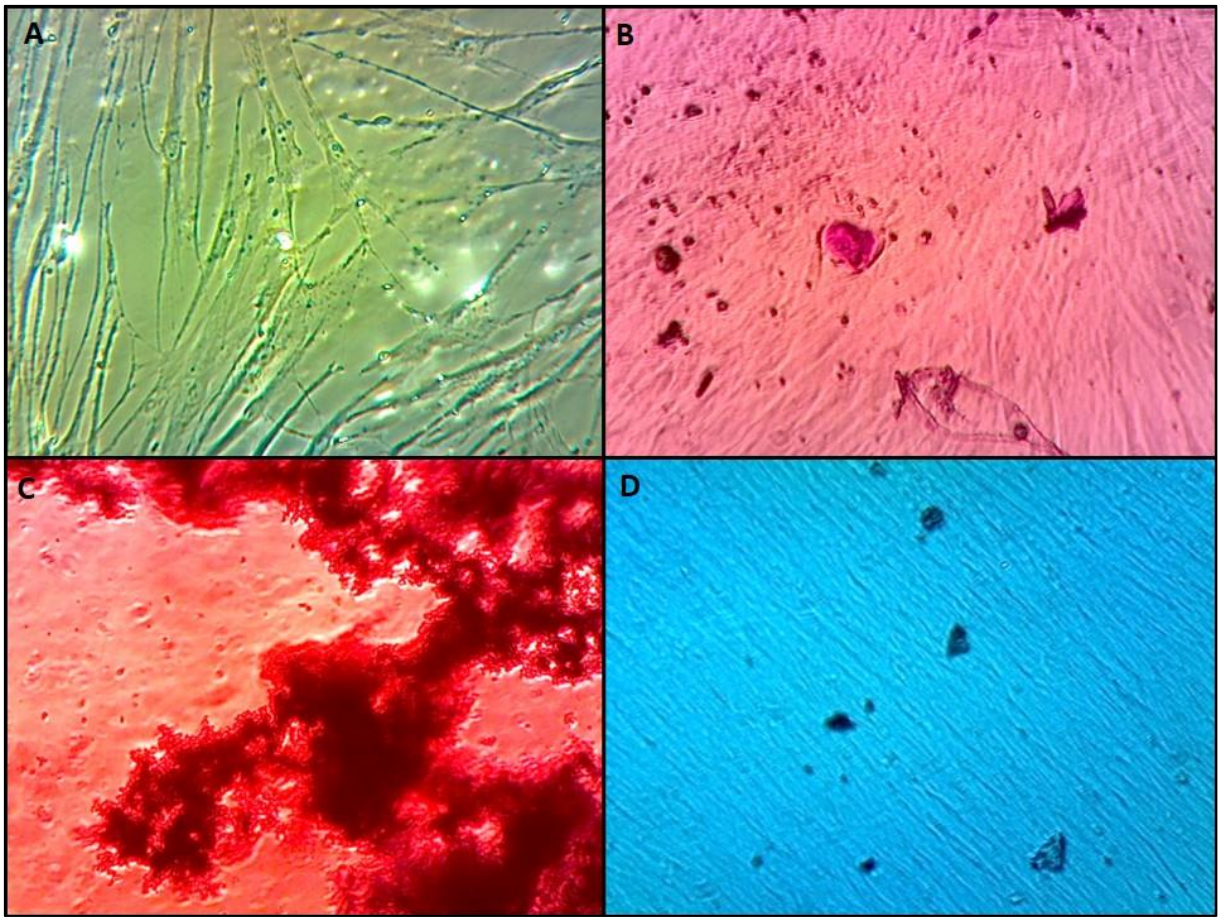

Figure 2: Characterization of hDPSCs in monolayer culture for their in vitro ability for osteogenic, adipogenic and chondrogenic differentiation. (A) hDPSCs cultured without induction of differentiation as negative control. (B) Calcium deposits stained with alizarin red indicating the osteogenic differentiation of hDPSCs. (C) Lipid vacuoles stained by Oil Red showing the adipogenic differentiation. (D) Chondrogenic differentiation visualized by Alcian blue staining of glycosaminoglycans deposits (original magnification, x100).

Photobiomodulation promotes increase in hDPSCs differentiation in 3D agarose model

Initially, a pilot study was performed to evaluate the differentiation capacity of hDPSCs cultured in 3D agarose model. As demonstrated in Figure 3, hDPSCs cultured in 3D after 14 days exhibited osteogenic (Figure 3A), adipogenic (Figure 3B), and chondrogenic differentiation (Figure 3C). These results indicated that agarose did not interfere with the differentiation properties of these stem cells and also in the absorbance measuring process.

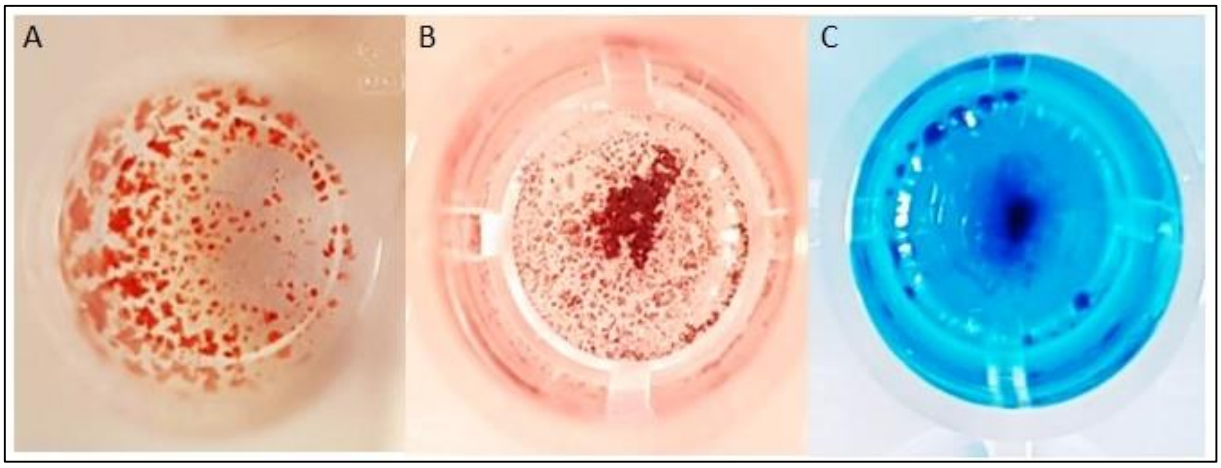

Figure 3: Macroscopic aspect of hDPSCs with osteogenic, adipogenic and chondrogenic differentiation in 3D agarose gel cultures. (A) Osteogenic differentiation visualized by calcium deposits stained with alizarin red. (B) Adipogenic differentiation observed by Oil Red staining lipid vacuoles. (C) Chondrogenic differentiation detected by Alcian blue staining glycosaminoglycans deposits (original magnification, well diameter = 6.4 mm).

After, the effect of PBM on the ability of hDPSCs to differentiate in a 3D culture model was evaluated (Figures 4, 5 and 6). PBM demonstrated positive effects, significantly stimulating both osteogenic, adipogenic and chondrogenic differentiation processes. All irradiated groups significantly increased the process of the three types of differentiation after 14 days ($p<0.05$).

Osteogenic differentiation was analyzed by calcium deposition after 7 and 14 days of cell culture in 3D and PBM treatment. After 7 days, only the PBM 5% FBS presented significant increase in osteogenic differentiation compared to control 10% FBS ($P<0.001$) (Figure 4A). At 14 days, both groups that received PBM (10% FBS and 5% FBS) exhibited significantly increased mineralization compared to controls ($p<0.001$) (Figure 4B).

The ALP assay in 3D culture confirmed the results of osteogenic differentiation following the same trend in enzyme activity. At 7 days, the highest activity for ALP enzyme was observed in FBM 5% FBS group compared with controls (10% and 5% FBS) (Figure 4C) ($p<0.05$). At 14 days, all FBM groups (10% and 5% FBS) presented significantly higher ALP compared to controls ($P<0.05$) (10% and 5% FBS) (Figure 4D).

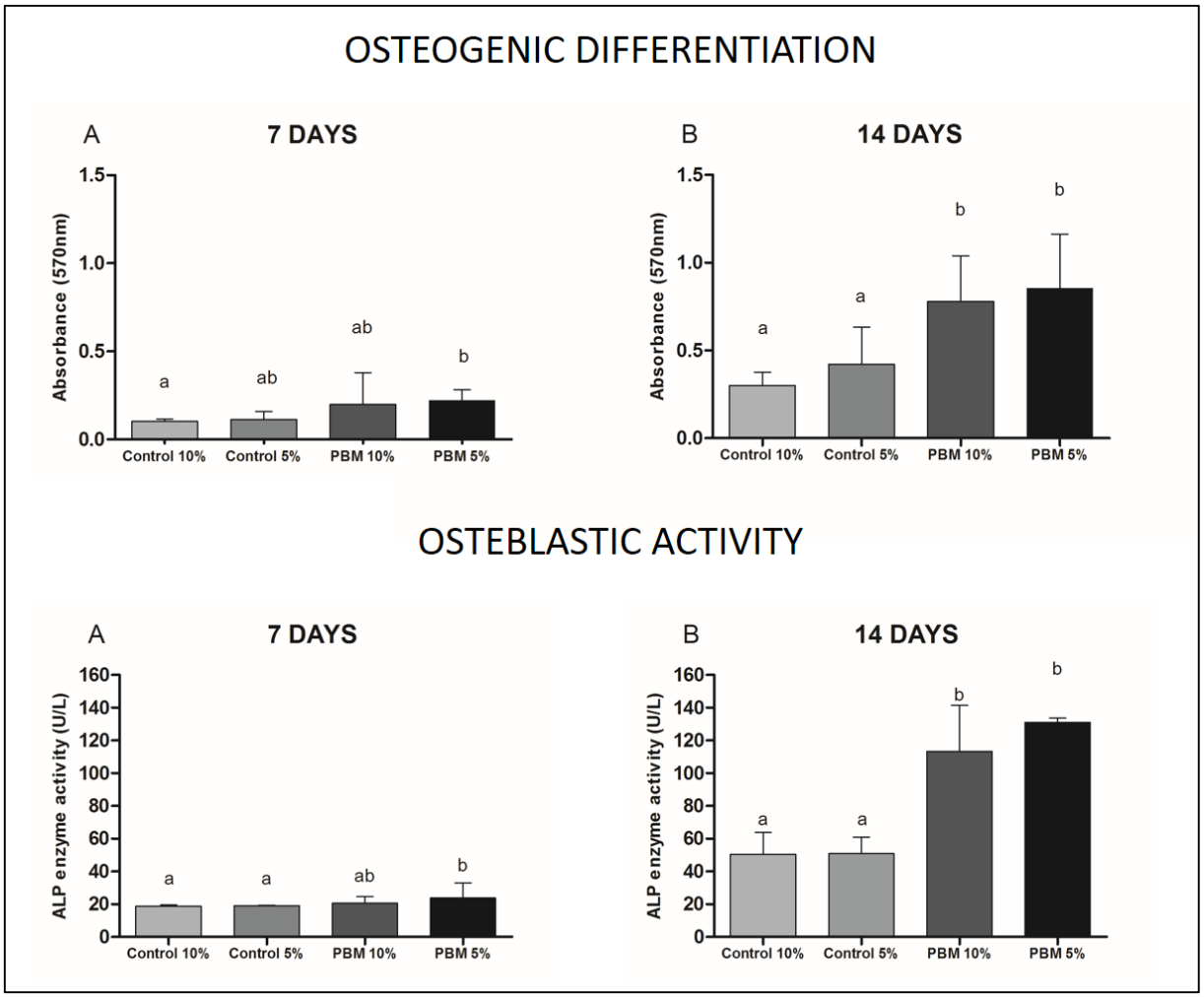

Figure 4: PBM promoted an increase in hDPSCs osteogenic differentiation (A and B) and osteoblastic activity (C and D) especially after 14 days in 3D agarose culture model. Bars with different letters represent significant differences between groups in each time point ($p<0.05$).

Regarding the analysis of adipogenic differentiation after 7 days, a decreased capacity was observed in control 5% FBS group compared to PBM groups (10% FBS and 5% FBS) (Figure 5A). However, at 14 days, all PBM groups (10% FBS and 5% FBS) showed significant increase in lipid vesicles formation compared to control groups (10% FBS and 5% FBS) ($P<0.05$) (Figure 5B).

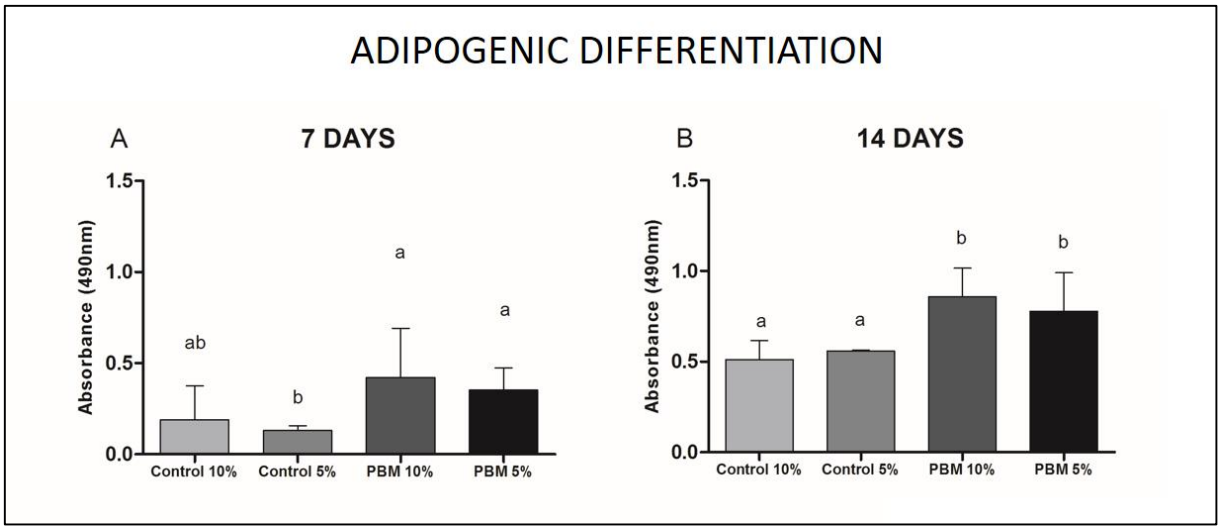

Figure 5: Adipogenic differentiation of hDPSCs after 7 and 14 days of 3D culture model. Both PBM groups (10%FBS and 5%FBS), after 14 days, showed significant increase of adipogenic differentiation compared to control groups (10%FBS and 5%FBS) ($p<0.05$). Bars with different letters represent significant differences between groups in each time point ($p<0.05$).

Chondrogenic differentiation evaluated by deposition of glycosaminoglycan was similar among groups at 7 days ($p>0.05$) (Figure 6A). At 14 days, PBM promoted an increase in deposition of these proteins compared only to the control 10% FBS ($p<0.05$) (Figure 6B).

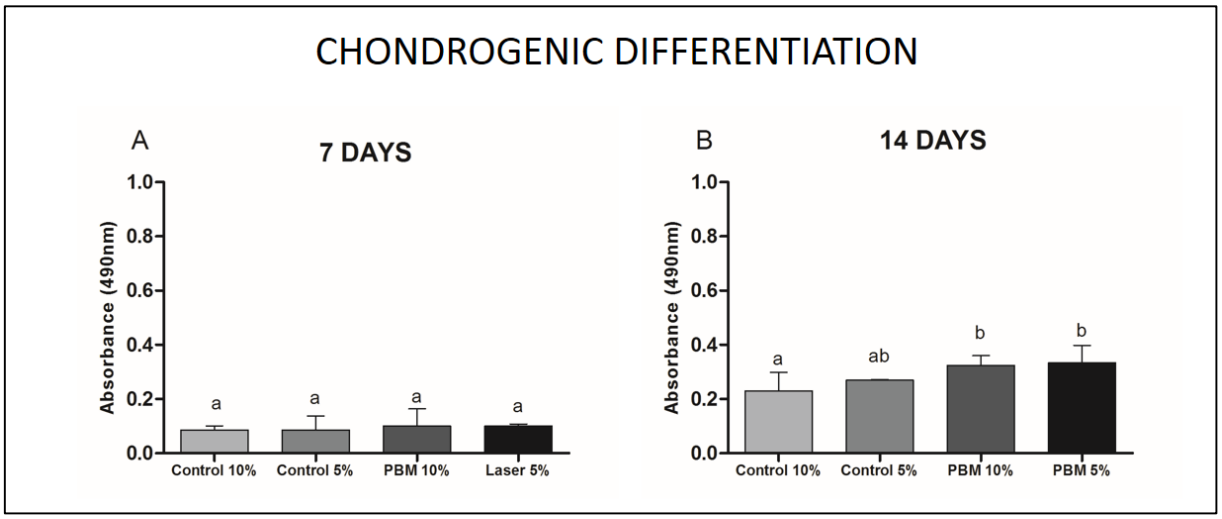

Figure 6: Chondrogenic differentiation of hDPSCs after 7 and 14 days evaluated by deposition of glycosaminoglycans. Only at 14 days, PBM promoted a significant increase in chondrogenic differentiation compared to the control 10% FBS ($p<0.05$). Bars with different letters represent significant differences between groups in each time point ($p<0.05$).

DISCUSSION

Mesenchymal stem cells are characterized by their high proliferative potential, regenerative capability and ability to differentiate in vitro into osteoblasts, chondrocytes, adipocytes, myocites and neural cells according to the microenvironment in which they are located (1, 25). A variety of tissues present mesenchymal stem cells (MSCs) that may be used in tissue engineering and regenerative medicine studies (1). Among them, the tooth is considered a rich source of these cells, identified by Gronthos et al. in 2000 (1) as dental pulp stem cells (DPSCs). Additionally, photobiomodulation (PBM) enhanced the proliferation and differentiation of DPSCs in monolayer cell culture, which does not accurately recreate the natural tissue architecture. Within that context, the identification of factors that enhance the proliferation and/or differentiation of DPSCs is extremely important. Thus, the present study analyzed (a) the capacity of hDPSCs to differentiate in three lineages using 3D agarose culture model and (b) the effect of PBM on the differentiation potential of hDPSCs in 3D agarose culture model under different nutritional conditions. The PBM demonstrated positive effects, significantly stimulating the osteogenic, adipogenic and chondrogenic differentiation process in 3D model.

Initially, hDPSCs were obtained and characterized by the combination of two methods, multilineage differentiation potential (osteogenic, adipogenic and chondrogenic) and by flow cytometry to assess the expression of STRO-1, CD44 and CD146 markers. These methodologies are corroborated by several studies that used the same markers of MSCs (1, 3, 4) and also by three multi-lineage differentiation potentials (5, 25). All these results were obtained using the monolayer culture (2D) model that has been used as standard in several *in vitro* studies with MSCs; however, some authors appoint that this arrangement of cells does not mimic the cell–cell interaction of tissues (26). Currently, 3D culture has become the best way to facilitate cell adhesion and proliferation, and it preserves the physiological function of tissue (13, 15, 27). Therefore, the 3D culture model could mimic the natural tissue architecture, facilitating analysis of the differentiation potential, since it is influenced by the surrounding microenvironment (13, 15, 27). Then, we decided to investigate if hDPSCs maintain the capacity of osteogenic, adipogenic and chondrogenic differentiation in three lineages using 3D culture model. Some previous cell culture

investigations used 3D agarose gel model with different concentrations ranging from 0.9 to 3% (28, 29, 30). Srikanth et al. in 2016 (30) demonstrated that a low concentration of agarose allows better understanding of the osteogenic differentiation when compared to higher concentrations. In the present study, we selected 0.3% agarose, that was a favorable concentration for cell adhesion and differentiation with easy cell manipulation during change of media and analyses steps. Our results showed that hDPSCs maintained the capacity of multi-lineage differentiation using 3D agarose culture model. These results are in accordance with some studies that demonstrated that synthetic and natural-based scaffolds, such as collagen sponges, chitosan, hydroxyapatite (HA), polymer, biomatrix or platelet lysate, have been used to load hDPSCs and showed that mineralized tissue can be formed when they are employed (27, 31, 32). Also, agarose has been considered a bioinert scaffold with non-toxic properties (17).

PBM is an emerging technique in which exposure to low-level laser light (red and near infrared radiation) or light emitting diodes (LED) stimulate cellular function by the activation of mitochondrial respiratory chain components, resulting in initiation of a signaling cascade that promotes activation of some signaling circuitries resulting in increased cellular metabolism and proliferation (18). Considering that PBM could allow a significant increase in viability, proliferation, migration and differentiation of stem cells, the 3D culture associated to its use may be a therapeutic opportunity in regenerative dentistry (8). Then, we tested the effect of PBM on the differentiation potential of hDPSCs in 3D agarose culture model with different nutritional conditions. The hDPSCs, in 3D agarose-based model and irradiated at the energy density tested in the present study, presented early mineral deposition in the cultures (7 days after induction).

Our main results showed that PBM (10% FBS and 5% FBS) promoted an increase in hDPSCs differentiation (osteogenic, adipogenic and chondrogenic), especially after 14 days, when compared with non-irradiated groups. These findings agree with previous investigations that analyzed the influence of PBM in osteogenic differentiation and ALP expression in monolayer culture using different irradiation parameters (33, 34). In contrast, other authors also evaluated the PBM effect in hDPSCs using different 3D cultures and observed that ALP activity increased until 7 days, but gradually decreased until 14 days (9, 27). These contradictory results could

be justified by biomaterials used or PBM protocols, including the different time intervals between each application used at the referred studies: 1-day interval, 30 cycles of 30 seconds only at first day, or 3-day intervals, respectively. Herein, the 6-hour interval was used because laser therapy exerts a dose-dependent effect on the biological responses and seems to have a cumulative effect at each new dose applied, and the best result of PBM has been observed in this time interval (22).

Some studies attributed the biostimulative effect of PBM on osteoblasts to an increased expression of autocrine factors that regulate osteoblast proliferation and differentiation, such as type 2 bone morphogenetic protein (BMP-2) and transforming growth factor (TGF)-β1 (9, 33, 34). These data suggest that PBM stimulates osteoblast differentiation in the initial proliferative and matrix synthesizing stages (48). Adipogenic differentiation was previously investigated to characterize the stem cells or evaluate some materials (15, 20, 25). However, until this moment, it was not used to quantify the lipid vesicle deposition induced by PBM. Similarly, in osteogenic differentiation and ALP activity, the adipogenic differentiation was increased in our study by PBM from 7 to 14 days ($P<0.05$).

The association of PBM and chondrogenic differentiation was previously evaluated by Kushibiki et al. in 2010 (35) using a prechondrogenic ATDC5 cells in monolayer culture. The authors observed that PBM enhanced the total collagen contents 14 days after low level laser irradiation ($P< 0.01$). Agreeing with them, the present investigation observed that hDPSCs induced chondrogenic differentiation in agarose gel, showing a significant increase in deposition of glycosaminoglycans when PBM was used in comparison with the control 10% ($p<0.05$). The present findings about the PBM biostimulation mechanisms on the adipocyte and chondrocyte expression genes and on the modulation of both differentiations must be further elucidated by new investigations.

With the intention to improve the effect of PBM on proliferation and differentiation, previous investigations demonstrated that a culture under nutritional deficit plays a positive influence on cell growth (6, 36). This happens because the nutritional deficit condition promotes an alteration of the redox state towards cell oxidation, and the cellular response to PBM is near optimal and stronger when potential redox is shifted (18, 37). Moreover, cells with lower pH are considered more sensitive to the laser action than those with neutral pH (6). The nutritional deficit aims

to simulate a clinical situation in which the tissue is in a stress process (38). Some studies evaluated the proliferation of, DPSCs, SHEDs and fibroblasts, respectively, under nutritional deficit of 5% FBS in monolayer culture (6, 36, 38). They found that irradiated cells in nutritional deficit presented cell growth similar or higher than the control cells in ideal nutritional condition. No studies have evaluated the effect of PBM in hDPSCs differentiation under nutritional deficit (5% FBS). Thus, the present study compared the osteogenic, adipogenic and chondrogenic differentiation under nutritional deficit or regular nutrition in 3D culture. However, there were no statistically significant differences between PBM groups with 5% or 10% FBS in all differentiations and ALP activity at 7 or 14 days ($p>0.05$). This may have occurred due to the employed PBM parameters or the different types of medium used by the referred studies.

CONCLUSION

Considering that PBM increased the bioactivity in this experimental model that mimics the natural tissue architecture and that this is a non-invasive and easy management technique, it can be concluded that the 3D agarose gel culture could be a promising scaffold in tissue engineering applications.

DISCLOSURE STATEMENT

No competing financial interests exist.

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Reprint Author

Patrícia M. P. Kopper, Av. Ramiro Barcelos, 2492, Santa Cecília, Porto Alegre-RS, Brazil; Telephone: +55 51 3308 5191; e-mail: patricia.kopper@ufrgs.br

5 CAPÍTULO 3

Influence of photobiomodulation therapy on root development: a study in rat molars with open apex and pulp necrosis.

I. M. Zaccara[1], L. B. Mestieri[1], A. P. Jardine[1], R. Quintana[1], L. Jesus[1], M. S. Moreira[2], F. S. Grecca[1], M. D. Martins[1], P. M. P. Kopper[1]

[1] Department of Conservative Dentistry, School of Dentistry, Federal University of Rio Grande do Sul - UFRGS, Porto Alegre, RS, Brazil.
[2] Department of Dentistry, School of Dentistry, University of Sao Paulo, Brazil; Ibirapuera University, Sao Paulo, Brazil.

Running Head: Influence of PBM therapy on root development.

Key Words: Dental pulp. Stem cells. Photobiomodulation therapy. Radicular development.

Correspondence author:
Patrícia Maria Poli Kopper
Division of Endodontics, Department of Conservative Dentistry, School of Dentistry, Federal University of Rio Grande do Sul - UFRGS, Porto Alegre, RS, Brazil.
Av. Ramiro Barcelos, 2492
Porto Alegre-RS, Brazil.
CEP: 90035-003
Telephone: 55 51 3308 5191
e-mail: pkopper@terra.com.br

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ABSTRACT

Aim: To evaluate, in vivo, the role of PBM in apexification and apexogenesis of rat molars with open apex and pulp necrosis using different protocols of treatment.

Methodology: Lower first molars of Wistar rats were exposed to oral environmental for 3 weeks. Then, the root canals were rinsed with 2.5% sodium hypochlorite and 17% EDTA, filled with an antibiotic paste and the teeth were sealed. After one week, the teeth were reopened, rinsed with the same solutions and divided into 6 groups (n=6): MTA; BC (blood clot); hDPSC; healthy tooth+PBM; MTA+PBM; BC+PBM; hDPSC+PBM. In hDSPC groups, 1% agarose gel scaffold was used for cell support. Two groups were not exposed to the oral environment: healthy tooth+PBM (n=6), healthy tooth (positive control, n=3); and one stayed exposed during the role experimental period: necrotic tooth (negative control, n=3). In PBM groups, irradiation was performed with InGaAlP laser, during 30 days, with 24-h intervals. After that, the rats were euthanized and specimens were processed for histological and immunohistochemistry analysis.

Results: Necrotic tooth presented greater neutrophil infiltrate than other groups ($P<0.05$). Necrotic tooth, healthy tooth and healthy tooth+PBM presented less fibrous condensation ($P<0.05$). All groups formed more mineralized tissue than necrotic tooth ($P<0.05$). PBM associated to MTA, BC or hDPSC formed more mineralized tissue than non-irradiated groups ($P<0.05$). MTA+PBM induced apexification ($P<0.05$). BSP marker confirmed the histological findings regarding mineralized tissue formation and hDPSCs groups exhibited higher percentage of BSP positive cells.

Conclusions: PBM improved the apexification and favors the initial stage of root canal complementation (apexogenesis) in teeth with pulp necrosis and open apex. Thus it could be recommended as an adjutant therapy to accelerate the apical healing and the root development process.

INTRODUCTION

Teeth with pulp necrosis and open apex have been traditionally treated with calcium hydroxide paste and, more recently, with mineral trioxide aggregate (MTA) (Torabinejad & Parirokh 2010, Camilleri *et al.* 2013, Nicoloso *et al.* 2017). These procedures aim to induce an apical barrier of hard tissue (apexification); however, there is no complementation of root formation, and the tooth may be weak and susceptible to fracture (Nicoloso *et al.* 2017). Therefore, the ideal treatment for necrotic immature permanent teeth would be the employment of tissue engineering procedures for the replacement of damaged tissues, favoring dentin and pulp regeneration, promoting the continuation of root development and tooth maintenance (Wang *et al.* 2013). Thus, knowledge on the biological processes involving the triad stem cells (SCs), scaffold (biomaterials), growth factors and other biostimulatory/biomodulatory procedures are necessary for an endodontic regenerative treatment (Murray *et al.* 2007, Marques *et al.* 2016).

Human dental pulp stem cells (hDPSCs) isolated from healthy teeth have shown proliferative potential and have been studied for several regenerative purposes, not only in dentistry but also in several medical applications (Gronthos *et al.* 2000, De Mendonça-Costa *et al*. 2008). These cells exhibit immunoregulatory capacity, suppressing the T cell immune response in vitro and in vivo, as in animal models (Jumah & Abumaree 2012). Based on that, SCs are good candidates for the treatment of mesenchymal tissue diseases and show a potential use in xenogeneic environment (Pierdomenico *et al*. 2005, Lin *et al*. 2012). Furthermore, it has been reported that the immunosuppressive properties of hDPSCs could make them attractive for use in animal transplants (Lin *et al*. 2012).

Stem cells need a structure to support and provide their viability when they are transplanted to another environment. In this context, biomaterials play a key role, providing a three-dimensional (3D) model to promote new tissue formation (van Hout *et al*. 2011). It may be used as scaffold for stem cell support and could also serve as synthetic microenvironments in terms of chemical composition, physical structure, and biological properties, affecting the proliferation and differentiation of stem cells (Szpalski *et al*. 2012, Piva *et al.* 2014). Nowadays, natural and synthetic scaffolds have been widely used in tissue engineering (Huang *et al.* 2010, Galler *et al.* 2011, Suzawa *et al.* 2015). Among natural scaffolds, the agarose gel (a polysaccharide of D-galactose and 3,6-anhydro-L-galactopyranose derived from the cell walls of red

algae) presents favorable characteristics considering transplantation, as being bioinert and non-toxic, and decreasing potential immune rejection in rats (Aurand *et al*. 2012, Pakulska *et al*. 2012, Suzawa *et al.* 2015).

According to the literature, photobiomodulation therapy (PBM) improves dentoalveolar-derived mesenchymal stem cells (ddMSCs), including hDPSCs, cell viability and proliferation and odonto/osteogenic differentiation. This therapy has been shown as promoting biostimulatory effects in vitro and in vivo in different cell types. It could stimulate cell growth, increase cell metabolism, improve cell regeneration, promote tissue response, and accelerate the regeneration of dentin after pulp exposure (Almeida-Lopes *et al*. 2001, Salate *et al*. 2005, Pretel *et al*. 2007, De Souza *et al.* 2011). Based on that, PBM therapy could be applied as a fourth element to improve the triad of tissue engineering (stem cells, scaffolds, and growth factors).

Considering the possibility to use tissue engineering for complementation of root development, it is justified to apply PBM, aiming to stimulate cell proliferation and differentiation accelerating dentin tissue and pulp cells regeneration. Therefore, the present study evaluated, in vivo, the role of PBM in apexification and apexogenesis of rat molars with open apex and pulp necrosis, using different protocols of treatment.

MATERIALS AND METHODS

The study was approved by the Institutional Review Board of the Federal University of Rio Grande do Sul, Brazil (CAAE 37252614.3.0000.5347) and Institutional Review Board for Animal Studies of the same University (27827), following the Brazilian guidelines for animal care and utilization for scientific purposes from the National Council of Animal Experimentation Control (CONCEA). Human DPSCs were obtained from four permanent third molars with incomplete root formation, indicated for extraction due to orthodontic reasons, from two healthy patients aged 18 years old. All participants signed an informed consent statement prior to any clinical procedure.

Animals

Twenty-six male rats (Rattus norvegicus albinus, rodentia mammalia—wistar lineage), 4 weeks years old, weighing 150 to 200g, were kept under standard conditions of temperature (20 to 24°C) and light/dark cycle, with solid chow and water ad libitum. Twenty-four animals were randomly divided into nine groups, according to treatment protocols for rat molars, and two were used for confirmation of experimental protocols (Figure 1).

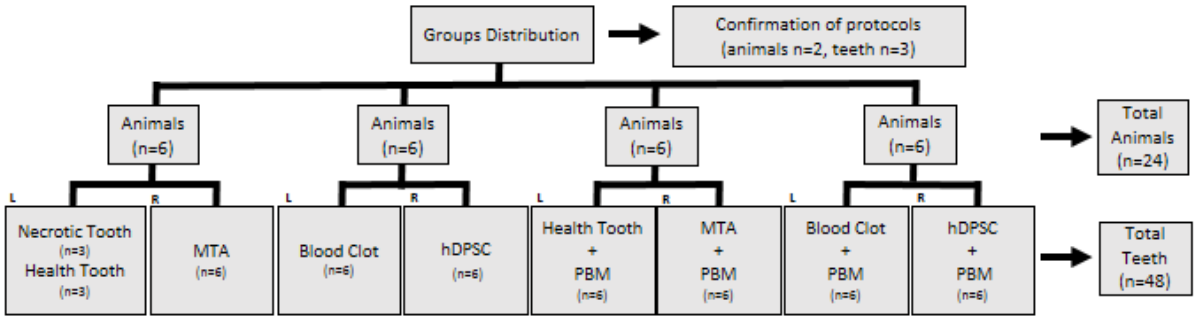

Figure 1: Flowchart illustrating group distribution according to the treatment protocols for rat molars. L: left tooth, R: right tooth. Necrotic tooth: pulp necrosis without treatment; Healthy tooth: vital pulp without treatment; MTA: pulp necrosis treated with MTA plug; Blood clot: pulp necrosis with blood clot induction; hDPSC: pulp necrosis with hDPSCs transplantation; Healthy tooth+PBM: vital pulp and PBM therapy; MTA+ PBM: pulp necrosis treated with MTA plug and PBM therapy; Blood clot + PBM: pulp necrosis with blood clot induction and PBM therapy; hDPSC+PBM: pulp necrosis with hDPSCs transplantation and PBM therapy.

Experimental procedures

Under aseptic conditions, the animals were anesthetized intraperitoneally with ketamine 80mg/kg and xylazine 20mg/kg (Syntec, Santana do Parnaíba, SP, Brazil). A device was used as previously described (Scarparo *et al.* 2011) to maintain the rats' mouths open. Dental pulps were exposed by drilling cavities on the central portion of the occlusal surface using a 1011HL bur (KG Sorensen, Cotia, SP, Brazil). The pulp tissues were removed with a #10 K-file (Dentsply Maillefer, Ballaigues, Switzerland) and the canals were left open for 3 weeks, time required for confirm pulp necrosis (Scarparo *et al*. 2011). After that, the root canals were irrigated with 2.5% sodium hypochlorite (NaOCl), followed by 17% EDTA, dried with absorbent paper points, filled with triple antibiotic, using an insulin syringe, and the teeth were sealed with amalgam. Antibiotic paste was composed by 50mg of each: metronidazole, ciprofloxacin, and minocycline per milliliter of propylene glycol paste.

Intracanal medication was maintained for 1 week (Hoshino *et al.* 1996). Thereafter, the coronal sealing was removed and the canals were irrigated with 2.5% NaOCl, followed by 17% EDTA.

The necrotic tooth group (negative control) included teeth that were left opened throughout the experimental period and that did not receive any treatment procedure. The healthy tooth group represented the positive control group and indicated that teeth were not submitted to any procedure. At MTA groups, the canals were filled with MTA plug in whole extension. At blood clot groups (BC), the apical foramens were overpassed and instrumented with a #10 K-file, promoting blood clot induction. At hDPSC groups, the apical foramens were also overpassed and instrumented, and then small blocks of agarose gel scaffold with the hDPSCs were transplanted into the canals. The groups Healthy tooth+PBM; MTA+PBM; Blood Clot+PBM; and hDPSC+PBM received 30 days of PBM therapy, as described below. After the procedures, all teeth from experimental and necrotic tooth groups were sealed with MTA and amalgam.

Two animals (n=3 teeth) were used to confirm necrosis induction, blood clot formation and transplantation of hDPSCs. For that, these animals were euthanized immediately after the procedures (Figure 2).

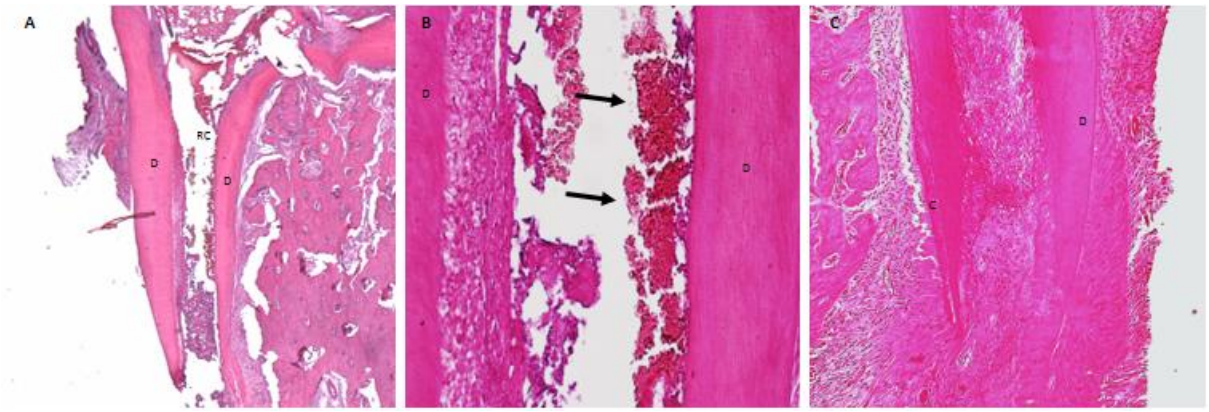

Figure 2: Histological aspects of teeth of animals euthanized immediately after the procedures to confirm the efficacy of protocols. (A) Root canal submitted to pulp removal procedure (H&E, 40x magnification). (B) Blood clot induction. Note the presence of blood cells within the canal (arrow) (H&E, 400x magnification). (C) Transplantation of hDPSC. D: dentin; RC: root canal; C: cementum. (H&E, 400x magnification).

Stem cell characterization and three-dimensional (3D) culture

For pulp tissue achievement, initially the apical papilla was removed. Then, the pulp was isolated from apical opening using Hedströem files (Maillefer, Dentsply, Switzerland) and stored in a culture dish (35x10 mm) containing Alpha Modification Minimum Essential Eagle's Medium (α-MEM, Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin and streptomycin (PenStrep, Gibco). The pulp tissues were sectioned into fragments of approximately 1 mm^3 and kept in supplemented α-MEM at 37°C, 95% humidity and 5% CO^2. The culture medium was changed 24h after pulp collection and at every 48h to obtain explants of hDPSCs. After reaching the confluence (70-90% of cells) of the plate, the cells were trypsinized (trysin, Sigma-Aldrich) to the next passage.

In the second passage (P2), the hDPSCs were analyzed to confirm their stem cell nature. Briefly, an aliquot of cells was evaluated by flow cytometry, which revealed positive staining for surface markers of mesenchymal stem cells (STRO1, CD146 and CD44) and negative staining for markers of hematopoietic stem cells (CD45 and CD14) (all from Santa Cruz Biotechnology, local). Additionally, the multilineage differentiation potential of hDPSCs, up to 14 days, was confirmed by culturing the cells in osteogenic, adipogenic and chondrogenic differentiation mediums.

A pilot study defined the ideal concentration of agarose gel for transplantation of hDPSC to the root canals of rat molars, leading to selection of a 1% agarose solution (Sigma-Aldrich, St. Louis, MO, USA) for preparation of 3D culture, using α-MEM. After preparation, 1000µL of this solution were pipetted into Petri dish. After agarose solidification, 1x106 of hDPSCs, cultured in α-MEM supplemented with 1% penicillin and streptomycin and regular 10% FBS, were seeded on the dish. Forty-eight hours after seeding, the areas with more quantity of cells in the scaffolds were selected to be transplanted inside the root canals of rat molars. The small blocks, with similar conformation of root canal, were transplanted within the canals of hDPSC and hDPSC+PBM groups.

Laser Irradiation

Considering that PBM therapy could have systemic effects, in the present study, PBM therapy groups were distributed in the same animals, avoiding the

interference of PBM on non-irradiated groups (Figure 1). Irradiation started immediately after each treatment using an InGaAlP diode laser (MMOptics, São Carlos, SP, Brazil), operating at the following parameters: wavelength of 660nm, power of 100mW, spot size of 0.3cm2, energy density of 3J/cm2, 10 seconds of exposure time, 1J of energy per point of application. Irradiation was performed on the buccal side of the tooth and was performed in 24-hour intervals, during 30 days, resulting in 30J of total energy in each tooth.

Histological Analysis

Animals were euthanized by overdose of isoflurane inhalant anesthesia (BioChimico, Itatiaia, RJ, Brazil) after 30 days of treatment procedures. Jaws were dissected for histologic and immunohistochemical evaluation.

Immediately after euthanasia, the specimens were fixed in buffered 10% formaldehyde for 24h, decalcified in Anna Morse solution (20% sodium citrate + 50% formic acid) for 20 weeks, rehydrated in ascending concentrations of ethanol, and embedded in paraffin. Three-micrometer sections were cut and stained with hematoxylin-eosin or processed for immunohistochemistry. The sections were selected according to visibility of root central portion, including the apex and periapical region.

The sections were observed under light microscopy (Olympus BX40 Microscope; Olympus Optical Co, Ltd, Tokyo, Japan). The features related to inflammatory infiltrate, fibrous condensation, apex conformation, deposition of mineralized tissue on the root canal walls and formation of mineralized barrier were evaluated by three blinded and calibrated examiners ($k = 0.89$, $P < .001$).

Inflammatory infiltrate cells were analyzed separately (neutrophil, lymphocyte, eosinophilic, macrophage and giant cells) and scored as absent (0), mild (1), moderate (2), and severe (3). The fibrous condensation was classified as absent (0), thin layer (1) or thick layer (2). The conformation of foraminal opening was classified as divergent (0) or convergent (1). The deposition of mineralized tissue on the root canal walls was scored as absent (0), thin layer (1) and thick layer (2), and the formation of mineralized barrier was scored as absent (0) or present (1).

Immunohistochemical analysis

The 3-µm sections were placed on silanized plates and heated in incubator for 30 minutes (min) at 80°C. After, they were deparaffinized in xylol, rehydrated in alcohol and PBS for 5 min. Antigenic recovery, endogenous peroxidases blocking and proteins blocking were made according to the following protocols: water bath for 35 min at 94°C in citrate buffer (pH=6.0); 5% hydrogen peroxide in absolute methanol (in the dark for 20 min); and 5% skimmed milk in PBS for 20 min, respectively. After, the sections were washed in cycles with distilled water and the last washing was made with PBS. Then, they were incubated with rabbit polyclonal anti-rat Bone Sialoprotein (BSP, 1:500; Abcam, Cambridge, CBE, United Kingdom) at 4-8°C overnight. Thereafter, the sections were washed 3 times in PBS and incubated with secondary conjugated antibodies and goat anti-rabbit IgG antibody (AP132p; Merck Milipore, Millerica, MA, USA), during 90 min, as recommended by the manufacturer. The sections were rinsed 3 times with PBS for 5 min and immunoreactivity was visualized after incubation with 3,30-diaminobenzidine (DAB) solution (Dako Liquid DAB, Substratehromogen System, Dako, Carpinteria, CA, USA), and then were counterstained with Hematoxylin. Immunohistochemical control was performed by replacing the primary antibodies with nonimmune serum. The positive controls for BSP were bone marrow.

The analysis of BSP immunoexpression along the root canals was performed describing the pattern of protein distribution on root walls as absent (0), thin layer (1), and thick layer (2), and the cells inside the root canal as absent (0), 0-50% of positive cells (1), and 50-100% of positive cells (2).

Data from histological and immunohistochemical analyses were compared by Kruskal-Wallis test followed by Dunn test. The significance level was set at 5%. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA).

RESULTS

Histological analyses

Histological analyses are summarized and illustrated on Figure 3 and Figure 4. The evaluation of the inflammatory cells revealed that necrotic tooth without treatment presented more pronounced neutrophil infiltrate in comparison to all other groups ($P<0.05$) (Figures 3A and 4A). Also, mild lymphocyte infiltrate was observed

in all groups (P>0.05) (Figure 3B). Eosinophil, macrophage and giant cells infiltrate were not observed.

Necrotic tooth, healthy tooth and healthy tooth+PBM presented thin layer or absence of fibrous condensation at the periapical area. All other groups stimulated the formation of a thicker layer of fibers (P<0.05) (Figure 3C). Regarding root apex conformation, all groups presented similar results (P>0.05) (Figure 3D). The analyses of mineralized tissue on the root canal walls showed that all groups formed more tissue than necrotic group (P<0.05). PBM associated with MTA, Blood clot and hDPSC induced more mineralized tissue formation compared to non-irradiated groups (P<0.05) (Figure 3E). MTA+PBM group was the only inducing apexification, being statistically different from all groups (P<0.05) (Figure 3F).

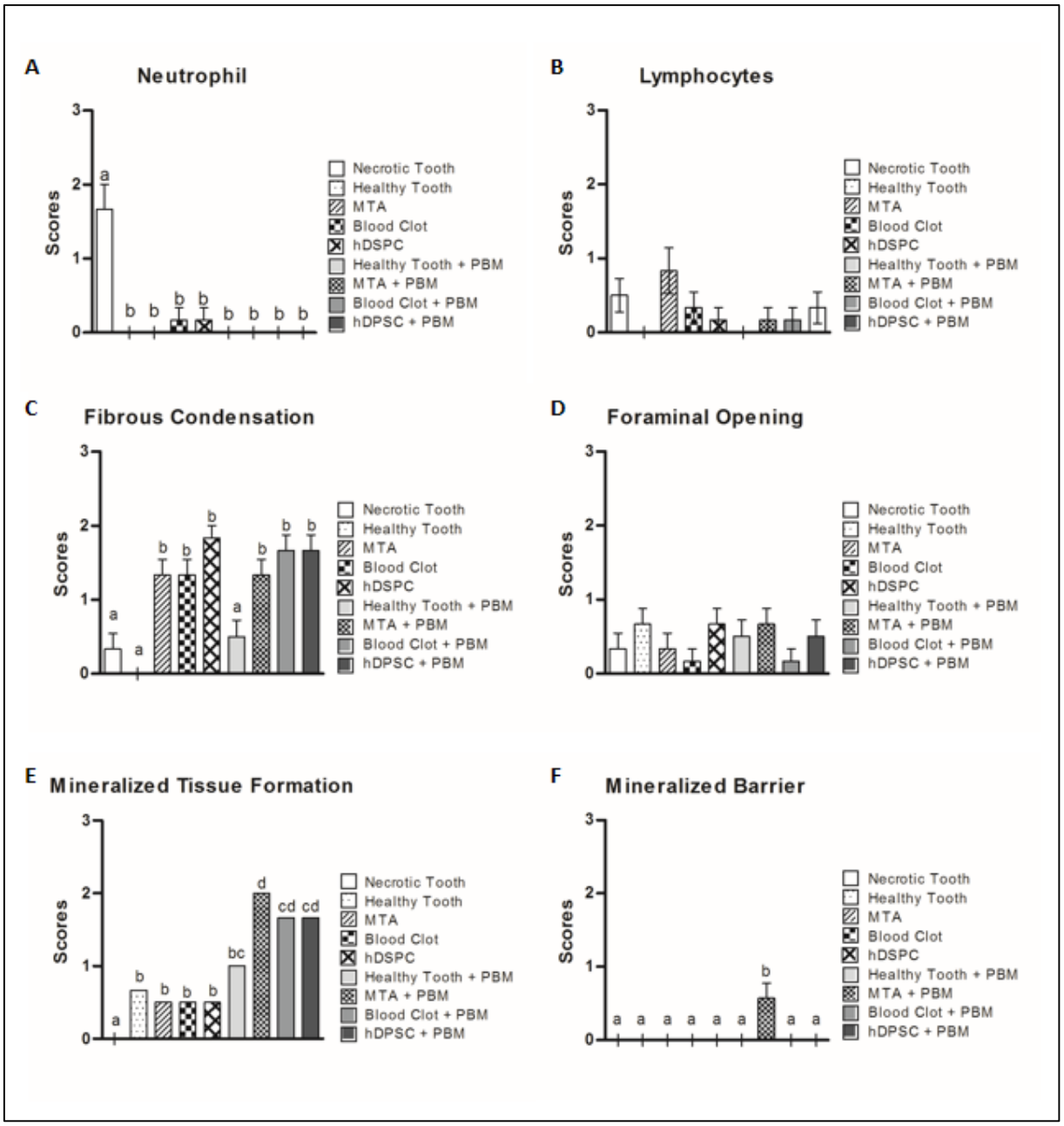

Figure 3: Graphic representation of the comparison between groups for (A) neutrophils, (B) lymphocytes, (C) fibrous condensation, (D) foraminal opening, (E) mineralized tissue formation, (F) mineralized barrier. Different lowercase letters indicate statistically significant difference ($P<0.05$).

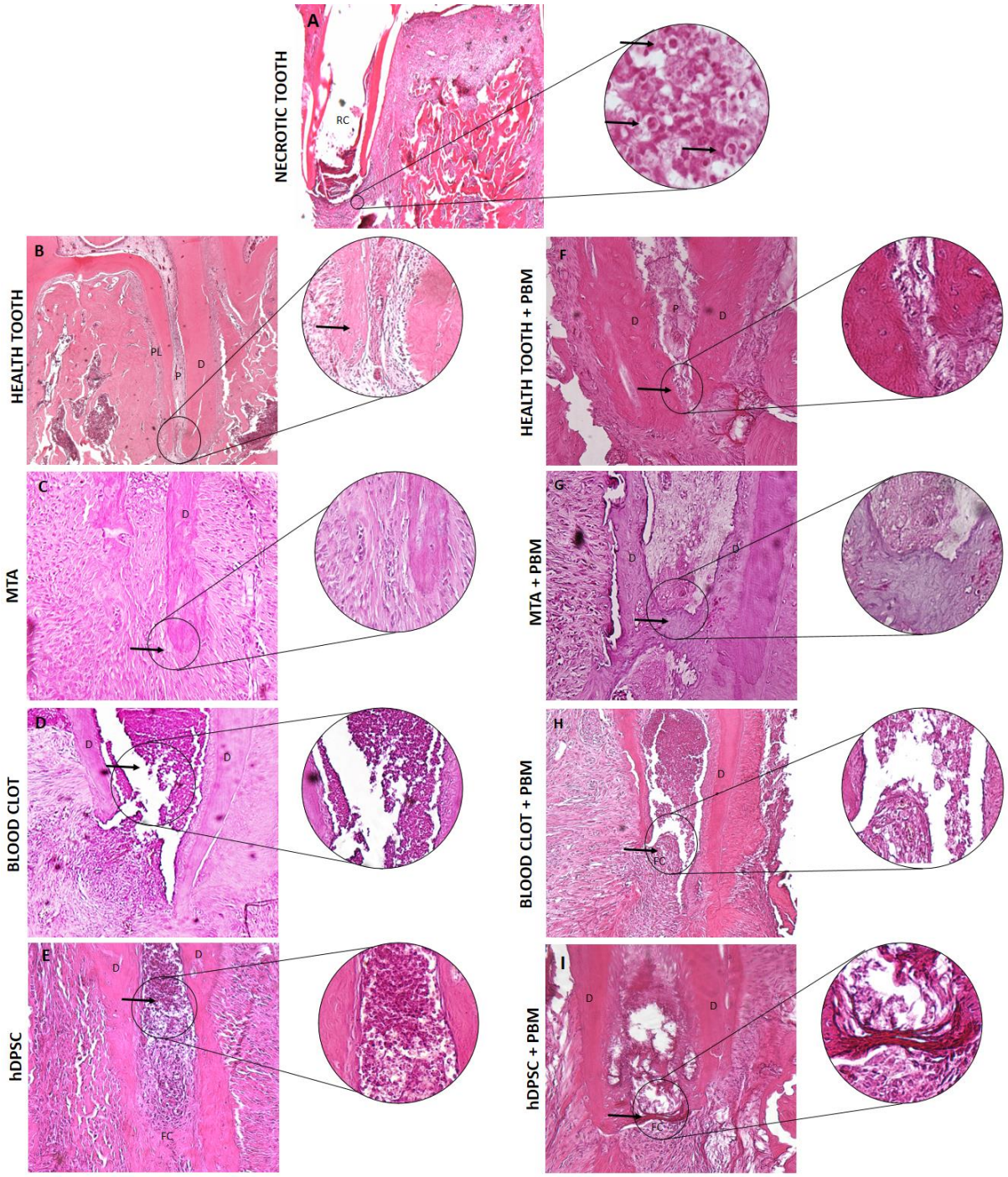

Figure 4: Histological aspects of all experimental groups. (A) Necrotic tooth group. Note moderate neutrophil infiltrate (arrows) (H&E, 40x magnification). (B) Healthy tooth group. Note natural root formation (arrow) (H&E, 40x magnification). (C) MTA group. Observe the thin layer of fibrous condensation at periapical area (H&E, 200x magnification). (D) Blood Clot group. Note cell concentration from blood clot in the root canal (arrow) (H&E, 200x magnification). (E) hDPSC group. Observe the presence of transplanted cells within the root canal. (F) Healthy teeth+PBM group. Natural root formation exhibiting complete radicular formation (arrow) (H&E, 200x magnification). (G) MTA+PBM group. Note the formation of mineralized barrier (arrow) (H&E, 200x magnification). (H) Blood clot+PBM group. Note the thin layer of fibrous condensation at periapical area (arrow) (H&E, 200x magnification). (I) hDPSC+PBM group. Note the presence of mineralized tissue in the root canal

walls (arrow) and thin layer of fibrous condensation at apical portion (asterisk) (H&E, 200x magnification). Disc shaped images represent higher magnification (1000 x (A) or 400x magnification). D: dentin; P: pulp; PL: periodontal ligament; RC: root canal; FC: fibrous condensation.

BSP immunostaining analyses

Figures 5 and 6 illustrate and summarize the immunoexpression of BSP. All groups showed positive reaction for BSP, which was more evident in the inner portion of root canal dentin. Necrotic tooth, healthy tooth, MTA, blood clot, hDPSC and healthy tooth+PBM groups showed a thin layer of BSP positive labeling on the root canal walls (Figures 5A, B, C, D, E, F and 6A). Meanwhile, when PBM therapy was applied concomitant with MTA, blood clot and hDPSC, a thicker layer of BSP positive labeling in root canal walls was noted ($P<0.05$) (Figures 5G, H, I and 6A). Besides that, MTA+PBM group exhibited complete apexification associated with thick BSP labeling in this new mineralized matrix (Figure 5G).

BSP analysis in cells inside the root canal revealed that hDPSC and hDPSC+PBM groups exhibited higher percentage (50-100%) of BSP positive cells than necrotic tooth, MTA and MTA+PBM ($P<0.05$). However, there were no differences from healthy tooth, blood clot, healthy tooth+PBM and blood clot+PBM groups ($P>0.05$) (Figures 5 and 6B).

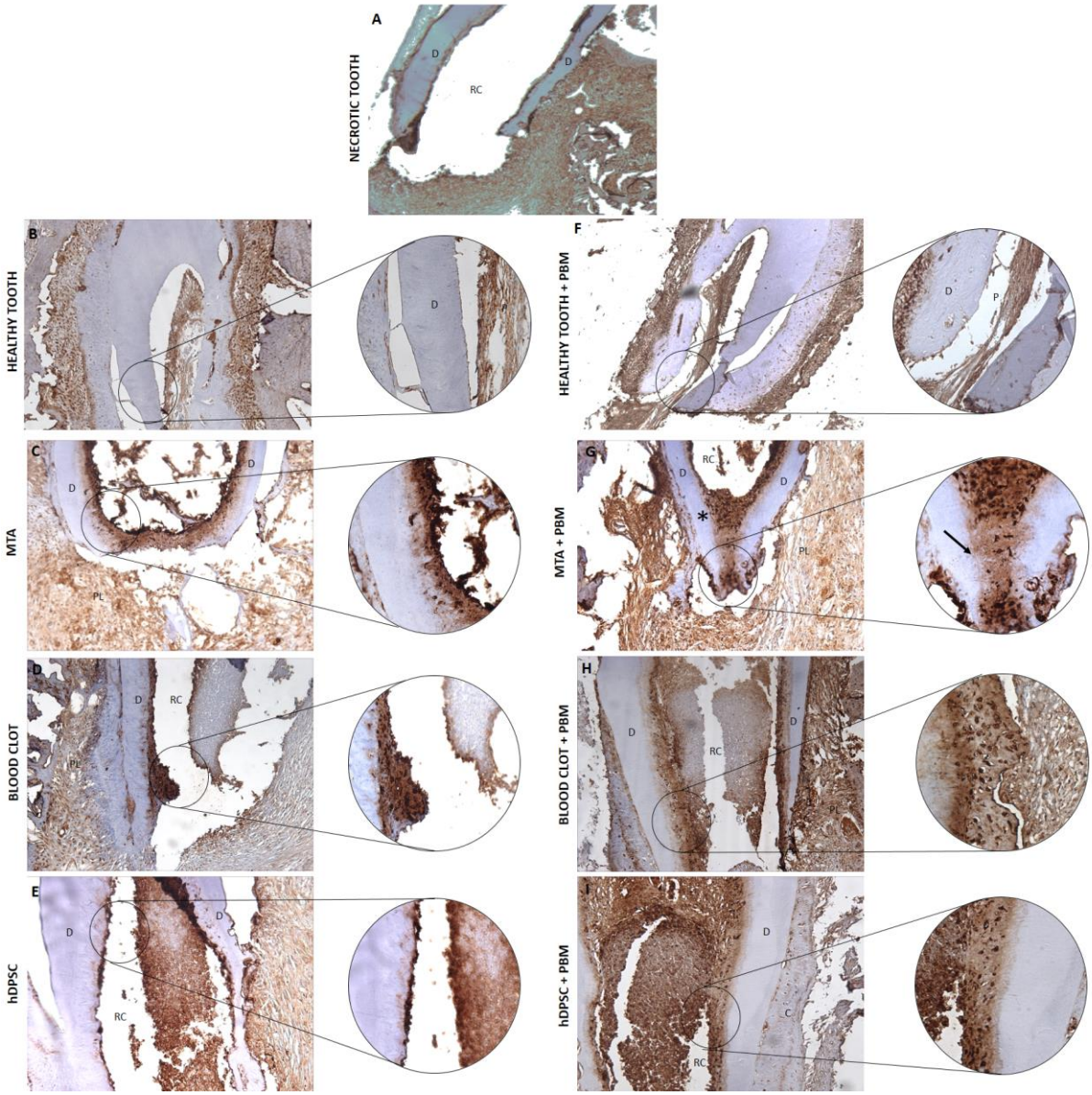

Figure 5: BSP immunoexpression of all experimental groups. Note the thin layer of BSP positive dentin in the inner portion of root canal walls at (A) Necrotic tooth group, (B) Healthy tooth group, (C) MTA group, (D) Blood clot group, (E) hDPSC group, (F) Healthy tooth+PBM group (HRP, 40x magnification). Observe the presence of thicker layer of positive BSP in the root canal walls at (G) MTA+PBM group, (H) Blood clot+PBM group, (I) hDPSC+PBM group (HRP, 40x magnification). Disc shaped images represent higher magnification (HRP, 400x magnification). D: dentin; P: pulp; PL: periodontal ligament; RC: root canal; FC: fibrous condensation.

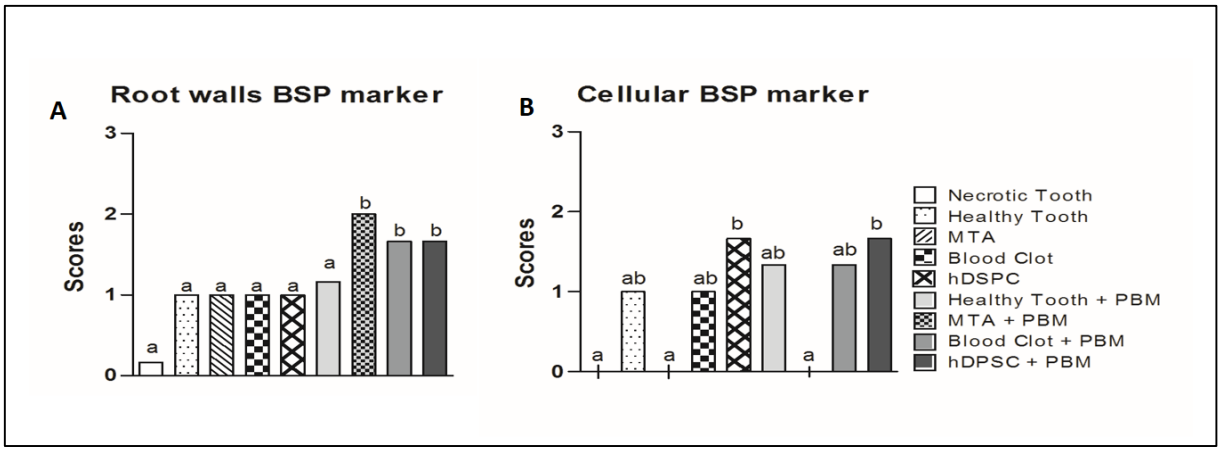

Figure 6: Graphic representation of the comparison between groups for immunoexpression of BSP marker for (A) Root walls BSP marker. (B) Cellular BSP marker. Different lowercase letters indicate statistically significant difference (P<0.05).

DISCUSSION

Tissue engineering and regenerative medicine have become important research areas (Galler *et al*. 2011). In endodontics, biological procedures using the triad of tissue engineering (stem cells + biomaterials + inductive growth and differentiation factors) have demonstrated potential to replace damaged structures, such as dentin and pulp complex (Murray *et al*. 2007, Hargreaves *et al.* 2013, Schmalz & Smith, 2014, Eramo *et al.* 2017). Recent in vitro studies demonstrated the effective influence of PBM in the proliferation and differentiation of SCs (Ginani *et al.* 2015, Marques *et al*. 2016, Borzabadi-Farahani 2016). However, in vivo, procedures using animal models are scarce. The present study was the first that evaluated apexification and apexogenesis in a model of induced pulp necrosis for long period associated with PBM therapy. The main results showed that PBM therapy improved dentin tissue formation when associated with MTA, blood clot and hDPSC in histological and immunohistochemical analyses.

MTA is considered a gold standard material for treatment of teeth with open apex and pulp necrosis, aiming to induce apexification (Nicoloso *et al.* 2017). It is an attractive material because it provides chemical signals for odontogenic/dentinogenic periapical tissue regeneration (Zhu *et al.* 2012, Nagy *et al.* 2014, Bonte *et al.* 2015). It occurs because, in the presence of blood or other biological fluid, they form hydroxyl and calcium ions during their hydration process. These ions act as chemical signals that alkalinize the microenvironment leading to new formation of mineralized tissue (Galdolfi *et al.* 2015). The current findings

showed that MTA group presented mineralized tissue formation similar to demonstrated by previous literature (Mah *et al.* 2003, Chueh *et al.* 2009, Nagy *et al.* 2014). Interestingly, PBM associated with MTA accelerated the process of mineralized tissue deposition (thicker layer) compared to MTA group. These results are in accordance with the studies of Fernandes *et al.* (2015) and Fekrazad *et al*. (2015), which showed positive effects of PBM associated with calcium hydroxide or MTA, respectively. Moreover, the present study observed that only MTA+PBM group was able to induce apical mineralized barrier formation in 30 days, being the first description of the effect of MTA and diode laser irradiation in tooth with pulp necrosis and open apex.

Regarding the promotion of apexogenesis, as an alternative approach, regenerative endodontics aims to repair dental pulp-like tissues using two possible strategies: cell homing and cell transplantation (Eramo *et al.* 2017). The blood clot (BC) is considered an important source of stem cells and could be used as a natural scaffold to support the in-growth of new tissue into the empty canal space. Several studies have shown that disinfection and blood clot formation can induce dentin deposition in root canal walls and increase the root length (Thibodeau *et al*. 2007, Zhu *et al.* 2012, Yang *et al*. 2015). This can occur because the blood clot is originated from the vascular component of periapical tissueand employs the host's endogenous cells to achieve tissue repair/regeneration, which is apotential for tissue regeneration (Zhu *et al.* 2012, Eramo *et al. 2017*). In addition, blood clot presents growth factors that stimulate the differentiation, growth, and maturation of fibroblasts, odontoblasts, and cementoblasts (Nagy *et al.* 2014). As previously described by the literature, in the present study, blood clot group showed mineralized tissue formation on the root canal walls (Thibodeau *et al.* 2007, Lovelace *et al.* 2011, Zhu *et al.* 2012).

Moreover,the combination of BC with PBM promoted a significant increase in mineralized tissue deposition. This association was previously investigated by Moreira *et al*. 2017; however, in their study, the protocol was used immediately after pulp removal of teeth without pulp necrosis induction, resulting in a dental pulp-like tissue with vessels, nerves, odontoblast-like cell layer and perivascular stem cells (Moreira *et al*, 2017). Furthermore, mineralized tissue formation along the root canal walls was not described. The present results call attention to cases of pulp necrosis and open apex, which are common in the endodontic clinic, showing that the use of

PBM associated with blood clot stimulation could promote the continuation of root development and consequently increase the chance of tooth maintenance.

As an alternative treatment alternative for apexogenesis, the transplantation of stem cells into the root canal can be promising. Some materials have already been used as scaffold to support the cells, aiming to analyze the regeneration of the dentin-pulp complex, such as HA/TCP, Gelfoam, synthetic polymer (PLG), synthetic hydrogel, collagen type I and III, platelet-rich plasma (PRP), silk fibroin protein and chitosan (Miura *et al.* 2003, Wang *et al.* 2013, *Huang* et al. 2010, Iohara *et al.* 2011, Zhu *et al.* 2012, Yang *et al.* 2015, Mangione *et al.* 2017, Palma *et al.* 2017). In the present study, hDPSCs was used associated with agarose scaffold due to the possibility to modify its consistency, according to the agarose concentration, becoming easy to manipulate and apply into the root canal. Agarose has already been used to regenerate osseous, cartilaginous and neural tissue (Aurand *et al*. 2012, Pakulska *et al.* 2012, Suzawa *et al.* 2015). However, this scaffold had never been used in root canals.

Furthermore, in this protocol, final root canal irrigation with 17% EDTA plays an important role. This solution induces the dentin to release growth factors that provide cell differentiation and expression of DSPP (dentin sialoprotein). It could be justified by the variety of growth factors that are present in the EDTA-soluble fraction that stays in direct contact with the demineralized human dentine extracellular matrix (Galler *et al.* 2016). The growth factors observed were transforming growth factor-b1 (TGF-b1), fibroblast growth factor-2 (FGF-2), bone morphogenetic protein-2 (BMP-2), platelet-derived growth factor (PDGF), placenta growth factor (PIGF) (Galler *et al.* 2016). Moreover, according to Zhang *et al.* (2011), these molecules are effective at very low concentrations and induce cellular responses, cell recruitment, proliferation and differentiation as well as mineralization.

Human DPSCs without PBM therapy promoted a thin layer of mineralized tissue along the root canal walls. Moreover, when hDPSC was associated with PBM therapy, the mineralized tissue deposition became thicker, improving the tissue regeneration along the root canal walls. Recent studies only demonstrated that the transplantation of hDPSCs into the root canal, without PBM therapy, promotes the formation of a vascularized pulp-like tissue, odontoblast-like cells, and newly generated dentin, without evaluating the mineralized tissue deposition (Bohl *et al*. 1998, Cordeiro *et al*. 2008, Huang *et al*. 2010). Herein the differentiation potential of

hDPSCs was confirmed by BSP marker, corroborating the previous findings of in vitro studies (Ohbayashi *et al.* 1999 Matsui *et al.* 2009). Moreover, the current investigation was the first that demonstrated, in vivo, the mineralized tissue deposition on root canal walls of teeth with pulp necrosis and open apex, associating transplantation of hDPSCs with PBM therapy.

In this scenario, PBM therapy increased the mineralized tissue formation when associated with MTA, blood clot and hDPSCs transplantation. Based on the present results, it could be inferred that PBM can be used as an adjutant treatment in teeth with pulp necrosis and open apex, aiming at apexification or apexogenesis. Some studies showed that PBM accelerates dentinogenesis and apexogenesis of healthy immature permanent teeth (Ferreira *et al.* 2006, Toomarian *et al.* 2012, Arany *et al.* 2014, Fekrazad *et al.* 2015). Furthermore, previous clinical studies demonstrated that PBM is an effective alternative for pulpotomy and pulp capping, presenting favorable tissue reactions and satisfactory tissue healing (Marques *et al*. 2014; Fernandes *et al.* 2015, Moreira *et al.* 2017). The positive effects of PBM in tissue mineralization can occur due to its capacity to improve cellular metabolism, increase cell proliferation and collagen synthesis, and favor angiogenesis, accelerating the healing process, and promoting analgesic and reparative effects (Almeida-Lopes *et al.* 2001, Salate *et al*. 2005, De Souza *et al.* 2011). In this context, in vitro studies have indicated the cumulative effect of applications of irradiation to promote cell proliferation, differentiation and mineralized tissue formation (Li *et al.* 2010, Soares *et al.* 2015, Zaccara *et al.* 2015). In addition, PBM increased the osteoblastic proliferation and gene expression of osteoblastic markers, such as alkaline phosphatase (ALP), runt-related transcription factor 2 (Runx-2), osteocalcin (OC), type I collagen (COL I), type III collagen (COL III), bone sialoprotein (BSP), transforming growth factor 2 (TGF-β), bone morphogenetic proteins (BMPs), and fibroblast growth factor (FGF) (Ferreira *et al.* 2006, Da Silva *et al.* 2012, Arany *et al.* 2014, Tim *et al*. 2015).

To confirm the potential of mineralized tissue formation in the experimental groups, the expression of BSP was verified. This marker is highly specific for mineralizing tissues, including bone, mineralizing cartilage, dentin, and cementum (Chen *et al.* 1993). The current findings showed that, when PBM therapy was applied concomitant with MTA, blood clot and hDPSC, a thicker layer of mineralized tissue marked positive for BSP. Complete apexification associated with strong BSP labeling

in the new mineralized matrix was also observed in MTA+PBM group. BSP labelling results corroborated the histological findings about mineralized tissue formation. Some previous studies also demonstrated that PBM improved bone formation and up-regulated the expression of some growth factors and genes related to bone cell differentiation, culminating in the stimulation of cell proliferation in the pulp-dentin complex (Pretel *et al.* 2007, Da Silva *et al.* 2012, Tim *et al.* 2015, Mangione *et al*. 2017). It is important to emphasize that BSP labelling was more evident in the inner portion of root canal dentin. This result is in accordance with Ganss *et al.* (1999), who stated that BSP plays an important role in the early stages of matrix formation and mineralization, making the mantle matrix dentin more evident than the tubular dentin.

CONCLUSION

PBM improved the apexification, with MTA, and favors the initial stage of root canal complementation (apexogenesis), with BC or hDPSC, in teeth with pulp necrosis and open apex. Thus it could be recommended as an adjutant therapy to accelerate the apical healing and the root development process. New clinical studies are important to validate the present results.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interests.

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6 CONSIDERAÇÕES FINAIS

A utilização da Terapia de Fotobiomodulação (PBM) na Odontologia vem se consolidando gradualmente a medida que novos estudos têm sido realizados. Apesar das diversificadas metodologias e parâmetros de irradiação utilizados, a literatura tem enfatizado os efeitos positivos promovidos pela PBM quando aplicada dentro de um intervalo aceitável de parâmetros que não danificam os fotorreceptores e também não causam efeitos deletérios para as células ou tecidos irradiados (ALGHAMDI, KUMAR, MOUSSA, 2012; EMELYANOV, KIRYANOVA, 2015; GINANI et al., 2015; BORZABADI-FARAHANI, 2016; MARQUES et al., 2016).

Neste sentido, a PBM demonstrou aumento da viabilidade das hDPSCs e esses achados estão de acordo com estudos anteriores (Eduardo et al., 2008, Soares et al. 2013, Zaccara et al., 2015, Moura-Netto et al., 2016). Além disso, vários autores demonstraram que a PBM tem um efeito dose-dependente nas respostas biológicas de acordo com o número de aplicações (Huang et al., 2009, Soares et al., 2013, Zaccara et al., 2015). O presente estudo também demonstrou que a PBM influencia significativamente a migração das hDPSCs, validando os resultados do MTT. Estes achados estão de acordo com estudos prévios in vitro (TSCHON et al., 2015; GAGNON et al., 2016) e in vivo, que comprovaram que a PBM desempenha um papel importante na melhora da cicatrização de feridas (PEPLOW, CHUNG, BAXTER, 2010; WAGNER et al., 2013)

Nesse contexto, a identificação de fatores que regulam o aumento da proliferação e diferenciação de hDPSCs é muito importante. Atualmente, estudos têm demonstrado que a epigenética regula o status de diferenciação e proliferação de CTs moduladas pelo equilíbrio entre as enzimas histona desacetilase (HDAC) e histona acetil transferases (HATs) (LEWIS et al. 2014; PAINO et al. 2014; SEO et al. 2015; DUNCAN et al 2016). Entretanto, o impacto da PBM em mecanismos epigenéticos em células-tronco mesenquimais (CTs), até o presente momento não havia sido descrito. Considerando os achados desta tese, pode-se inferir que a PBM desempenha um papel semelhante aos inibidores de HDAC, promovendo modificações nucleares, que induz o aumento da acetilação de histonas, intensificando a expressão gênica e, desta forma, justificando o efeito biostimulador da PBM nos ensaios de viabilidade e migração aqui avaliados.

A PBM aumentou a diferenciação de hDPSCs em estudos que utilizaram cultura de células em monocamadas (YAMADA, CUKIERMAN, 2007). Porém, esta condição não recria com precisão a arquitetura do tecido natural. Os resultados da presente investigação mostraram que as hDPSCs mantiveram a capacidade de diferenciação em um modelo de cultura 3D em gel de agarose. Tais achados concordam com os encontrados em estudos prévios que também empregaram modelos de cultura 3D, como esponjas de colágeno, quitosana, hidroxiapatita ou polímeros sintéticos (ABRAMOVITCH-GOTTLIB et al., 2005; YANG et al. 2011; ABUARQOUB et al. 2015).

O aumento das diferenciações osteogênica, adipogênica e condrogênica, após 14 dias, com o uso da PBM aqui observado, está de acordo com investigações anteriores que analisaram sua influência na diferenciação osteogênica e condrogênica e na atividade da ALP, em cultura monocamada, usando diferentes parâmetros de irradiação (MEDINA-HUERTAS et al. 2014; MANZANO-MORENO et al. 2015; KUSHIBIKI et al., 2010). No entanto, Abramovitch-Gottlib et al. (2005) e Theocharidou et al. (2017), empregando diferentes culturas 3D, observaram que a atividade ALP aumentou até 7 dias, mas diminuiu gradualmente até 14 dias. Estes resultados contraditórios podem ser justificados pela diferença nos biomateriais ou devido aos prolongados intervalos de tempo entre cada aplicação do laser. De acordo com Meneguzzo et al. (2008), o efeito da PBM é potencializado quando utilizada em intervalos de tempo de 6 horas.

Atualmente, a engenharia de tecidos se tornou uma área bastante pesquisada (GALLER et al., 2011). Além disso, na área da Endodontia, torna-se importante investigar protocolos, que utilizam a combinação de princípios da engenharia de tecidos com o uso de células-tronco, biomateriais (*scaffolds*) e fatores de crescimento, que possam favorecer o reparo radicular de dentes com necrose pulpar e rizogênese incompleta (MURRAY, GARCIA-GODOY, HARGREAVES, 2007; HARGREAVES, DIOGENES, TEIXEIRA, 2013; SCHMALZ, SMITH, 2014). Atualmente, já foi avaliado o efeito estimulador da PBM no desenvolvimento de raízes de dentes com rizogênese incompleta que foram pulpotomizados imediatamente após sua exposição, apresentando cicatrização tecidual satisfatória nos dentes irradiados (MARQUES et al., 2015, FERNANDES et al., 2015). Estudos prévios também observaram uma maior taxa de dentinogênese e apexogênese de dentes permanentes hígidos irradiados (TOOMARIAN et al., 2012; FEKRAZAD et al.,

2015). No presente estudo, foi analisado o tratamento de primeiros molares inferiores necrosados de ratos, associando o uso da PBM a protocolos que visam estimular a apicificação, com uso de MTA, ou apicigênese, através da indução de coágulo sanguíneo ou implantação de hDPSCs em gel de agarose (*scaffold*). A escolha do gel de agarose como *scaffold* foi devido a suas características que favorecem a implantação de células: não é tóxico e diminui o potencial de rejeição imune em ratos (SUZAWA et al., 2015; ABUELBA et al., 2015). Foi observado que os grupos irradiados apresentaram maior formação de tecido mineralizado comparados com os não irradiados. Além disso, a associação do MTA com a PBM induziu a apicificação em 30 dias de análise. Além disso, a avaliação imunohistoquímica do marcador BSP confirmou os achados histológicos relacionados a deposição de tecido mineralizado nas paredes internas dos canais radiculares e ratificou o potencial de diferenciação osteogênica das hDPSCs.

Sendo assim, nas referidas situações clínicas, tais protocolos podem ser considerados como uma alternativa de tratamento. Além disso, a presente investigação foi a primeira que avaliou in vivo a influência da PBM no reparo radicular de dentes necrosados com rizogênese incompleta, demonstrando resultados favoráveis associados aos diferentes protocolos de apicificação (MTA) e apicigênese (coágulo sanguíneo ou transplante de hDPSCs) e, desta forma, influenciando positivamente no processo de reparo e no desenvolvimento radicular.

Pode-se concluir, pela análise dos resultados descritos nos três artigos apresentados na presente tese, que a PBM desempenhou um importante papel no aumento da viabilidade e da migração que estão relacionados com o aumento da regulação epigenética das células, promovendo o aumento da acetilação das histonas e, dessa forma, aumentando a expressão gênica. Além disso, a PBM aumentou a diferenciação das referidas células em cultura 3D. Sendo assim, a PBM pode ser considerada uma alternativa de tratamento em situações clínicas de dentes permanentes com necrose pulpar e rizogênese incompleta. Além disso, o modelo de gel de agarose mostrou ser um *scaffold* promissor em aplicações de engenharia de tecidos. A realização de novos estudos clínicos será importante para validar os resultados do presente estudo.

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ANEXOS

ANEXO A- Carta de Aprovação pela Comissão de Pesquisa da Universidade Federal do Rio Grande do Sul (COMPESQ) do projeto in vivo.

UFRGS

Universidade Federal do Rio Grande do Sul — Faculdade de Odontologia

PARECER CONSUBSTANCIADO DA COMISSÃO DE PESQUISA

Parecer aprovado em reunião do dia 19 de setembro de 2014

ATA nº 10/2014.

A Comissão de Pesquisa da Faculdade de Odontologia da Universidade Federal do Rio Grande do Sul após análise aprovou o projeto abaixo citado com o seguinte parecer:

Tendo em vista que o tratamento ideal para dentes permanentes imaturos necrosados é o desenvolvimento de procedimentos biológicos para reposição dos tecidos danificados, tal como a indução da formação do tecido dentinário e as células do tecido pulpar, favorecendo a manutenção do dente e a complementação do desenvolvimento radicular normal, tornam-se necessárias pesquisas envolvendo células-tronco, biomateriais e engenharia de tecidos. Objetivos: O objetivo deste estudo será avaliar o comportamento de quatro protocolos para tratamento de molares de ratos com rizogênese incompleta e necrose pulpar, na indução da complementação da formação radicular. Materiais e Métodos: Serão avaliadas quatro terapias em primeiros molares inferiores de ratos da linhagem Wistar com rizogênese incompleta que tiveram seus canais expostos ao meio bucal por 3 semanas para a indução da necrose pulpar: indução de coágulo, plasma rico em plaquetas (PRP), associação de células da polpa dentária humana a membrana PRP e associação de células da polpa dentária humana ao scaffold quitosana/colágeno. Quatro e sete semanas após os procedimentos experimentais será feita a eutanásia dos animais (n=14 por período, 12 dos grupos experimentais e 2 controles). Os dentes serão analisados radiograficamente, histologicamente quanto a intensidade da resposta inflamatória, e imunoistoquímica, por meio da marcação de STRO-1, CD44, DSPP, CAP e BMP-2. Os dados obtidos serão submetidos ao teste de Kolmogorov-Smirnov para determinar o padrão de distribuição e selecionar o teste estatístico mais indicado e o nível de significância será de 0,05.
O projeto encontra-se bem descrito e com mérito científico. Portanto somos pela aprovação. Os autores devem encaminhar o projeto ao comitê de ética de uso em animais, cadastrá-lo na Plataforma Brasil para posterior encaminhamento ao comitê de ética.

PROJETO: 27827 - ANÁLISE DA INDUÇÃO DA COMPLEMENTAÇÃO DA FORMAÇÃO RADICULAR DE MOLARES DE RATOS NECROSADOS COM RIZOGÊNEZE INCOMPLETA

PESQUISADOR RESPONSÁVEL: PATRICIA MARIA POLI KOPPER MORA

Porto Alegre, 19 de setembro de 2014.

Juliana Jobim Jardim
Professora - UFRGS
CRO-RS 12929

Profa. Dra. Juliana Jobim Jardim
Coordenadora da
Comissão de Pesquisa ODONTOLOGIA UFRGS

Porto Alegre RS Rua Ramiro Barcelos, 2492 CEP 90035-003 Fones 0xx51- 33 08 51 67

ANEXO B – Parecer de Aprovação pelo Comitê de Ética em Pesquisa da Universidade Federal do Rio Grande do Sul (CEP-UFRGS) do projeto in vivo.

UFRGS
UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL / PRÓ-REITORIA DE PESQUISA -

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Análise da indução da complementação da formação radicular de molares de ratos necrosados com rizogêneze incompleta

Pesquisador: Patrícia Maria Poli Kopper Móra

Área Temática:

Versão: 3

CAAE: 37252614.3.0000.5347

Instituição Proponente: Universidade Federal do Rio Grande do Sul

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 938.457

Data da Relatoria: 14/01/2015

Apresentação do Projeto:

Trata-se de um projeto de pesquisa que será desenvolvido junto ao PPG da Faculdade de Odontologia da UFRGS.

Objetivo da Pesquisa:

Objetivo Geral:

O objetivo deste estudo será avaliar, in vivo, o comportamento de quatro protocolos para tratamento (indução de coágulo; plasma rico em plaquetas – PRP; associação de células da polpa dentária humana ao scaffold PRP; e associação de células da polpa dentária humana ao scaffold quitosana/colágeno) de molares de ratos, com rizogênese incompleta e necrose pulpar induzida, na indução da complementação da formação radicular.

2.2 Objetivo Específico:

Proposição Específica 1: avaliar o comportamento clínico e radiográfico de molares de ratos com rizogênese incompleta e necrose pulpar submetidos a quatro protocolos de tratamento para indução da complementação radicular.

Proposição Específica 2: avaliar a resposta tecidual de molares de ratos com rizogênese incompleta e necrose pulpar submetidos a quatro protocolos de tratamento para indução da complementação radicular.

Endereço: Av. Paulo Gama, 110 - Sala 317 do Prédio Anexo 1 da Reitoria - Campus Centro
Bairro: Farroupilha **CEP:** 90.040-060
UF: RS **Município:** PORTO ALEGRE
Telefone: (51)3308-3738 **Fax:** (51)3308-4085 **E-mail:** etica@propesq.ufrgs.br

Proposição Específica 3: imunolocalizar a presença de células troncos no canal radicular de molares de ratos com rizogênese incompleta e necrose pulpar submetidos a quatro protocolos de tratamento para indução da complementação radicular, através dos marcadores STRO-1 e CD44.
Proposição Específica 4: imunolocalizar a deposição de tecido calcificado no canal radicular de molares de ratos com rizogênese incompleta e necrose pulpar submetidos a quatro protocolos de tratamento para indução da complementação radicular, através dos marcadores CAP, BMP2 e DSPP.

Avaliação dos Riscos e Benefícios:
As ponderações com relação aos riscos e benefícios atendem às recomendações das normativas vigentes.

Comentários e Considerações sobre a Pesquisa:
Inicialmente, serão incluídos na pesquisa quatro dentes terceiros molares humanos hígidos com formação radicular incompleta de pacientes diferentes que pode ser de ambos os sexos, independente de raça, com idade entre 15 e 18 anos, em bom estado de saúde sistêmica e bucal e que deseje participar doando o elemento dentário mediante assinatura, por seu responsável, de Termo de Consentimento Livre e Esclarecido (TCLE) – anexo 1. Os dentes terceiros molares deverão ter indicação cirúrgica e/ou ortodôntica de exodontia, sem cárie, restauração ou doença periodontal. Em caso de intercorrência que impossibilite o uso do elemento dentário (ex. fratura do dente durante exodontia) será considerado perda da pesquisa e novo indivíduo será convidado a doar seu dente nas mesmas condições. Os terceiros molares humanos servirão de fonte de células-tronco para posterior implantação nos canais radiculares de 28 ratos machos com quatro anos de idade, sendo 6 ratos por grupo.
Será avaliado, in vivo, o comportamento de quatro protocolos para tratamento de molares de ratos, com rizogênese incompleta e necrose pulpar, na indução da complementação da formação radicular. Para tal, serão avaliadas quatro terapias em primeiros molares inferiores de ratos da linhagem Wistar com rizogênese incompleta que tiveram seus canais expostos ao meio bucal por 3 semanas para a indução da necrose pulpar: indução de coágulo, plasma rico em plaquetas (PRP), associação de células da polpa dentária humana ao scaffold PRP e associação de células da polpa dentária humana ao scaffold quitosana/colágeno. Quatro e sete semanas após os procedimentos experimentais os animais serão submetidos a eutanásia (n=14 por período, 12 dos grupos experimentais e 2 controles). Os dentes serão

analisados radiograficamente e, logo após, será feita análise histológica, da intensidade da resposta inflamatória, e imunoistoquímica, por meio da marcação de STRO-1,CD44, DSPP, CAP e BMP-2.

Considerações sobre os Termos de apresentação obrigatória:

- Carta de ciência e concordância do laboratório onde o estudo será realizado foi devidamente apresentada.
- Folha de rosto, parecer COMPESQ, orçamento e cronograma foram incluídos.
- Os pesquisadores apresentaram justificativa para o número de ratos utilizados e para o número de dentes a partir dos quais serão obtidas as células tronco.
- Os pesquisadores prestaram esclarecimento a respeito do recrutamento dos participantes.
- A linguagem do TCLE e do Termo de Assentimento foi modificada e está mais ao alcance do público.

Recomendações:

Sem recomendações adicionais.

Conclusões ou Pendências e Lista de Inadequações:

Considerando que as recomendações do CEP foram atendidas, o projeto encontra-se em condições de ser aprovado.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Aprovado.

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL / PRÓ-REITORIA DE PESQUISA -

Continuação do Parecer: 938.457

PORTO ALEGRE, 26 de Janeiro de 2015

Assinado por:
MARIA DA GRAÇA CORSO DA MOTTA
(Coordenador)

ANEXO C – Parecer de aprovação pelo Comitê de Ética em Pesquisa com Animais (CEUA-UFRGS)

U F R G S
UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

PRÓ-REITORIA DE PESQUISA

Comissão De Ética No Uso De Animais

CEUA UFRGS

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 27827

Título: ANÁLISE DA INDUÇÃO DA COMPLEMENTAÇÃO DA FORMAÇÃO RADICULAR DE MOLARES DE RATOS NECROSADOS COM RIZOGÊNEZE INCOMPLETA

Pesquisadores:

Equipe UFRGS:

PATRICIA MARIA POLI KOPPER MORA - coordenador desde 13/08/2014
FABIANA SOARES GRECCA VILELLA - pesquisador desde 13/08/2014
DAIANA ELISABETH BOTTCHER - Aluno de Doutorado desde 13/08/2014
Ivana Maria Zaccara Cunha Araújo - Aluno de Doutorado desde 13/08/2014
Leticia Boldrin Mestieri - Aluno de Doutorado desde 13/08/2014

Comissão De Ética No Uso De Animais aprovou o mesmo , em reunião realizada em 27/10/2014 - Sala I do Gabinete do Reitor - Prédio da Reitoria - Campus do Centro - Porto Alegre, em seus aspectos éticos e metodológicos, para a utilização de 28 ratos Wistar machos, de acordo com as Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008 que disciplina a criação e utilização de animais em atividades de ensino e pesquisa.

Porto Alegre, Segunda-Feira, 10 de Novembro de 2014

STELA MARIS KUZE RATES
Coordenador da comissão de ética

ANEXO D- Termo de Consentimento Livre e Esclarecido (TCLE) do projeto in vivo.

UFRGS
UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE ODONTOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Convidamos você para participar da pesquisa sobre **"Análise da indução da complementação da formação radicular de molares de ratos necrosados com rizogêneze incompleta"** que tem por objetivo avaliar o potencial de regenerar tecidos das células-tronco da polpa dental (interior do dente), o que pode no futuro contribuir para o avanço no tratamento de várias doenças.

A sua participação terá risco mínimo, nada além daquele que o procedimento cirúrgico para extração dentária oferece, como inchaço, dor e sangramento. Você não será submetido a nenhum procedimento (extra) além da cirurgia de remoção do dente, que pode ter sido indicada porque o dente não erupcionou (dente não nasceu), por razões ortodônticas ou para prevenir reabsorção das raízes do dente vizinho. Nós utilizaremos nesta pesquisa apenas terceiros molares (sisos) que estejam sadios, porém você deve autorizar os pesquisadores a examinarem os seus fragmentos teciduais (dentes removidos), que seriam tirados de sua boca e desprezados após a cirurgia. Depois da remoção da polpa dental, seu dente será descartado de modo adequado (lixo biológico), seguindo os mesmos procedimentos das extrações convencionais. Depois de realizar procedimentos para avaliar o potencial regenerativo das células desta polpa, todo material será descartado, assim não haverá armazenamento de material biológico. Todas as informações confidenciais serão guardadas em local seguro e somente usadas com o propósito científico, sem divulgação do seu nome.

Sua participação é voluntária, caso queira, você poderá desistir a qualquer momento, sem que isso lhe traga prejuízo ou penalidade, basta que retire o seu consentimento em participar.

A pesquisa deverá contribuir para o aumento do conhecimento no campo interdisciplinar onde princípios de engenharia e ciências biológicas são utilizados para o desenvolvimento de materiais que podem substituir tecidos e recuperar a função do ser vivo. A importância desta pesquisa, portanto, está no fato da necessidade do desenvolvimento e aprimoramento, assim como, um melhor entendimento para facilitar na escolha de tratamentos mais eficazes em regeneração de tecidos, trazendo assim, benefícios para a sociedade de um modo geral.

Você não terá nenhum gasto financeiro por qualquer procedimento executado por essa pesquisa e terá direito a reembolso (ressarcimento) de qualquer gasto comprovadamente que você tenha feito para a realização desse estudo, bem como será indenizado em caso de dano comprovadamente ocorrido por sua participação na mesma.

Você receberá uma cópia desse termo no seu endereço via correio com aviso de recebimento e qualquer dúvida a respeito da pesquisa poderá perguntar a Patrícia Maria Poli Kopper Móra, no Campus Saúde no Departamento de Odontologia Conservadora da Faculdade de Odontologia da Universidade Federal do Rio Grande do Sul, Santana, Porto Alegre/RS, fone (51) 3308-5191, celular (51) 8177-8127 e E-mail: patrícia.kopper@ufrgs.br.

Consentimento Livre e Esclarecido

Declaro que compreendi os objetivos desta pesquisa, como ela será realizada, os riscos e benefícios envolvidos e concordo em participar voluntariamente da pesquisa **"Análise da indução da complementação da formação radicular de molares de ratos necrosados com rizogêneze incompleta".**

Assinatura ou impressão

Digital do responsável do voluntário: _____

Data:___/___/___

Assinatura do pesquisador: _____

Data:___/___/___

Quanto à ética dessa pesquisa poderá ser questionada ao Comitê de Ética em Pesquisa da Universidade Federal do Rio Grande do Sul (CEP/UFRGS) , pelo telefone (51) 3308-3738.

ANEXO E – Carta de Aprovação pela Comissão de Pesquisa da Universidade Federal do Rio Grande do Sul (COMPESQ) do projeto in vitro.

UFRGS

Universidade Federal do Rio Grande do Sul — Faculdade de Odontologia

PARECER CONSUBSTÂNCIADO DA COMISSÃO DE PESQUISA

Parecer aprovado em reunião do dia 15 de maio de 2015

ATA nº 05/2015.

A Comissão de Pesquisa da Faculdade de Odontologia da Universidade Federal do Rio Grande do Sul após analise aprovou o projeto abaixo citado com o seguinte parecer:

Prezado Pesquisador PATRICIA MARIA POLI KOPPER MORA,
Informamos que o projeto de pesquisa 28814 - ANÁLISE DA INFLUÊNCIA DO LASER DE BAIXA INTENSIDADE NO POTENCIAL DE PROLIFERAÇÃO E DIFERENCIAÇÃO DE CÉLULAS-TRONCO DA POLPA E DA PAPILA DENTAL DE DENTES PERMANENTES encontra-se aprovado com o seguinte parecer:

O objetivo deste estudo será avaliar o efeito da irradiação do LBI na proliferação e diferenciação de células-tronco da polpa e papila dental de dentes permanentes hígidos. Materiais e Métodos: Extratos de polpa e papila dental serão isolados de quatro terceiros molares hígidos removidos por indicação cirúrgica e/ou ortodôntica. As células serão caracterizadas e cultivadas em meio de cultura αMEM suplementado com antibióticos e 10% de soro fetal bovino. No terceiro subcultivo, as DPSCs e SCAPs serão divididas de acordo com o grupo a que pertencer (controle positivo – 10%FBS, controle negativo – 2%FBS, grupo teste 2 J/cm2, grupo teste 4 J/cm2). Nos grupos testes um laser diodo InGaAlP (modelo Twin Laser) será utilizado com comprimento de onda de 660nm, potência de 40mW, variando densidades de energia (2 e 4 J/cm2). Serão feitas aplicações do laser nos intervalos de 24 horas, durante 4 dias, para análise de proliferação, e 14 dias para análise de diferenciação. A fim de avaliar a proliferação celular, serão utilizados o método MTT e Alamar Blue, nos intervalos de 24, 48 e 72h após a primeira aplicação do laser. Para avaliação da diferenciação, as células serão cultivadas em meios indutores (osteogênico e adipogênico) e após 7 e 14 dias será quantificada a formação de matriz mineraliza e vacúolos lipídicos, respectivamente, através de espectrofotometria. Os dados obtidos serão submetidos ao teste de Kolmogorov-Smirnov para determinar o padrão de distribuição e selecionar o teste estatístico mais indicado e o nível de significância será de 0,05. O presente projeto possui mérito, encontra-se bem descrito e os autores responderam adequadamente as solicitações requeridas. Sendo assim, somos favoráveis à aprovação do projeto, devendo o mesmo ser incluído no sistema Plataforma Brasil para apreciação Ética.
Atenciosamente, Comissão de Pesquisa
Comissão de Pesquisa em Odontologia

Porto Alegre, 15 de maio de 2015.

Fabrício Collares
Professor - UFRGS
CRO-RS 15048

Prof. Dr. Fabrício Mezzomo Collares
Coordenador da
Comissão de Pesquisa ODONTOLOGIA UFRGS

ANEXO F- Parecer de Aprovação pelo Comitê de Ética em Pesquisa da Universidade Federal do Rio Grande do Sul (CEP-UFRGS) do projeto in vitro.

UFRGS UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL / PRÓ-REITORIA DE PESQUISA -

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Análise da influência do laser de baixa intensidade no potencial de proliferação e diferenciação de células-tronco da polpa e da papila dental de dentes permanentes.

Pesquisador: Patrícia Maria Poli Kopper Móra
Área Temática:
Versão: 2
CAAE: 45459615.8.0000.5347
Instituição Proponente: Universidade Federal do Rio Grande do Sul
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.173.546
Data da Relatoria: 30/07/2015

Apresentação do Projeto:
Trata-se de um projeto relacionado ao estudo do efeito do laser de baixa intensidade (LBI), o qual tem sido utilizado com a finalidade de promover cicatrização e regeneração dos tecidos, mas que foi pouco estudado em células-tronco dentais, como polpa (DPSC) e papila dental (SCAP).

Objetivo da Pesquisa:
Objetivo Primário:
O presente estudo tem como objetivo avaliar, através de experimentos in vitro, a influência do laser de baixa intensidade sobre o potencial de proliferação e diferenciação de células-tronco da polpa e papila dental de terceiros molares permanentes humanos hígidos.

Objetivo Secundário:
Proposição Específica 1: Caracterizar as células-tronco da polpa e da papila dental de terceiros molares permanentes humanos hígidos;

Proposição Específica 2: Avaliar a influência do laser de baixa intensidade sobre a viabilidade celular das células-tronco da polpa e da papila dental de dentes terceiros molares hígidos, através dos métodos de Ensaio de MTT e Alamar Blue;

Endereço: Av. Paulo Gama, 110 - Sala 317 do Prédio Anexo 1 da Reitoria - Campus Centro
Bairro: Farroupilha **CEP:** 90.040-060
UF: RS **Município:** PORTO ALEGRE
Telefone: (51)3308-3738 **Fax:** (51)3308-4085 **E-mail:** etica@propesq.ufrgs.br

Proposição Específica 3: Avaliar a influência do laser de baixa intensidade sobre a capacidade de diferenciação osteogênica das células-tronco da polpa e da papila dental de dentes terceiros molares hígidos,nos períodos de 7 e 14 dias;

Proposição Específica 4: Avaliar a influência do laser de baixa intensidade sobre a capacidade de diferenciação adipogênica das células-tronco da polpa e da papila dental de dentes terceiros molares hígidos, nos períodos de 7 e 14 dias;

Avaliação dos Riscos e Benefícios:

Riscos e benefícios foram devidamente apresentados.

Comentários e Considerações sobre a Pesquisa:

Amostra: quatro dentes terceiros molares humanos hígidos com formação radicular incompleta de pacientes com idade entre 15 e 18 anos, dos quais será obtida quantidade suficiente de células. 2.Isolamento e obtenção da cultura celular: Imediatamente após a exodontia, a papila dental será coletada, mediante a separação cuidadosa da região apical com o auxílio de descolador de Molt. A polpa dentária será coletada via abertura apical

dos dentes com auxílio de instrumento Hedströen (Maillefer, Dentsply, Chemin du Verger, Ballaigues, Suíça) compatível com a abertura radicular, realizando movimentos de rotação e tração para remoção do tecido.

Em seguida, os tecidos serão armazenados em placa de Petri 35x10 mm contendo meio de cultura -MEM completo (meio de culura -MEM suplementado com 10% de soro fetal bovino – FBS e 1% de penicilina e streptomicina), mantidos em condições hipotérmicas (em isopor contendo gelo) para transporte até o fluxo laminar do Laboratório de Cultura de Células da FO-UFRGS, onde será adequadamente processado. Dentro do fluxo, o tecido coletado será seccionado em fragmentos de aproximadamente 1 mm3 com auxílio de uma lâmina de bisturi. Estes serão mantidos em estufa a 37°C, 95% de umidade e 5% de CO_2 até se observar a migração das células do tecido para a placa de Petri 35 x 10mm, onde serão mantidos até atingirem 70 a 90% de confluência, com troca de meio a cada três dias. Em seguida, passagens sucessivas serão realizadas para expansão celular. No

subcultivo o meio básico será removido, 2 ml de tripsina/EDTA (0,25% de Tripsina contendo 1mM de EDTA – Cultilab, Brasil) serão adicionados para desprendimento das células das placas.

A suspensão celular será então colocada em um tubo cônico com o mesmo volume de meio -MEM suplementado com 10% de FBS com o objetivo de inativar a tripsina. A suspensão será centrifugada a 1200 rpm durante 8 minutos, o sobrenadante será retirado, as células ressuspensas em 1ml de meio -MEM, e então no terceiro subcultivo (P3) as células serão utilizadas nos

experimentos. Para a criopreservação celular, será utilizado o protocolo sugerido por Ding et al. (2010), utilizando 90% de FBS e 10% de dimetil-sulfóxido (DMSO),
diminuindo a toxicidade do DMSO. A maior quantidade de FBS deve proporcionar mais nutrientes para a preservação da estrutura celular e reparação após o descongelamento. Alíquotas de 1,0 mL de células (2x105 células/ mL) em meio de congelamento serão transferidas para criotubos e congeladas a -80ºC em recipiente para congelamento celular gradativo (Mr. Frosty, Nalgene, Rochester, NY, USA). 3.Caracterização: Em citômetro
de fluxo, será analisada a presença de CD146, CD44 e STRO1 e análise da ausência de CD34. Após atingirem a confluência, aproximadamente 5x103 células serão incubadas a 4°C por 30 minutos com os anticorpos descritos previamente (diluição de 1:10) para a marcação das moléculas de superfície. Decorrido este período, as células serão lavadas com
PBS e fixadas com solução específica (paraformaldeído 1%, azida sódica 0,1%, e 0,5% de FBS) para posterior analise em citômetro de fluxo. 4.Delineamento do Estudo:Na terceira passagem (P3), as células serão divididas acordo com os parâmetros de LBI para análises de proliferação ou diferenciação:Grupo Controle Positivo (10% FBS);Grupo Controle Negativo (2%FBS);Grupo 1: 2J/cm2 (2%FBS);Grupo 2: 4J/cm2:(2%FBS); Grupo 3: 2J/cm2: (10% FBS); Grupo 4: 4J/cm2: (10% FBS). 5.Aplicação do LBI: Laser diodo InGaAlP (Twin Laser,
MM Optics, São Paulo, Brasil)aplicado em intervalo de 24 horas durante 4 dias. O medidor de energia LaserCheck será utilizado para verificar a potência do laser de
saída do equipamento. A irradiação será realizada parcialmente no escuro a fim de evitar a influência de outras fontes de luz, em modo de aplicação contínuo e pontual (uma aplicação por poço), posicionando-se a ponta do laser diretamente no fundo das placas de cultura. O grupo controle será tratado nas mesma condições.

Considerações sobre os Termos de apresentação obrigatória:

- Parecer da COMPESQ/ODO, folha de rosto, termo de assentimento, termo de doação de dentes e TCLE foram apresentados e encontram-se em condições de aprovação.
- Os pesquisadores argumentam que o documento anexado previamente (Laboratório de Cultura) caracterizava ciência e concordância do responsável pelo Laboratório onde será realizado o experimento. Embora o referido documento caracterize a solicitação da pesquisadora e não a concordância manifesta do responsável, é possível a aprovação uma vez que o professor que assina o documento é o responsável pelo laboratório. Essa informação a respeito do professor não havia sido oferecida anteriormente.

Recomendações:

Sem recomendações adicionais.

Conclusões ou Pendências e Lista de Inadequações:

O projeto encontra-se em condições de aprovação.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Aprovado.

PORTO ALEGRE, 06 de Agosto de 2015

Assinado por:
José Artur Bogo Chies
(Coordenador)

ANEXO G – Termo de Consentimento Livre e Esclarecido (TCLE) do projeto in vitro.

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE ODONTOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Convidamos você para participar da pesquisa sobre **"Análise da influência do laser de baixa intensidade no potencial de proliferação e diferenciação de células-tronco da polpa e da papila dental de dentes permanentes"** que tem por objetivo avaliar o efeito do laser no crescimento das células-tronco da polpa dental (interior do dente), o que pode no futuro contribuir para o avanço no tratamento de várias doenças.

A sua participação terá risco mínimo, nada além daquele que o procedimento cirúrgico para extração dentária oferece, como inchaço, dor e sangramento. Você não será submetido a nenhum procedimento (extra) além da cirurgia de remoção do dente, que pode ter sido indicada porque o dente não erupcionou (dente não nasceu), por razões ortodônticas ou para prevenir reabsorção das raízes do dente vizinho. Nós utilizaremos nesta pesquisa apenas terceiros molares (sisos) que estejam sadios, porém você deve autorizar os pesquisadores a examinarem os seus fragmentos teciduais (dentes removidos), que seriam tirados de sua boca e desprezados após a cirurgia. Depois da remoção da polpa dental, seu dente será descartado de modo adequado (lixo biológico), seguindo os mesmos procedimentos das extrações convencionais. Depois de realizar os testes para avaliar como as células do dente reagem ao laser, todo material será descartado, assim não haverá armazenamento de material biológico. Todas as informações confidenciais serão guardadas em local seguro e somente usadas com o propósito científico, sem divulgação do seu nome.

Sua participação é voluntária, caso queira, você poderá desistir a qualquer momento, sem que isso lhe traga prejuízo ou penalidade, basta que retire o seu consentimento em participar.

A pesquisa deverá contribuir para o aumento do conhecimento no campo interdisciplinar onde princípios de engenharia e ciências biológicas são utilizados para o desenvolvimento de materiais que podem substituir tecidos e recuperar a função do ser vivo. A importância desta pesquisa, portanto, está no fato da necessidade do desenvolvimento e aprimoramento, assim como, um melhor entendimento para facilitar na escolha de tratamentos mais eficazes em regeneração de tecidos, trazendo assim, benefícios para a sociedade de um modo geral.

Você não terá nenhum gasto financeiro por qualquer procedimento executado por essa pesquisa e terá direito a reembolso (ressarcimento) de qualquer gasto comprovadamente que você tenha feito para a realização desse estudo, bem como será indenizado em caso de dano comprovadamente ocorrido por sua participação na mesma.

Você receberá uma cópia desse termo no seu endereço via correio com aviso de recebimento e qualquer dúvida a respeito da pesquisa poderá perguntar a Patrícia Maria Poli Kopper Móra, no Campus Saúde no Departamento de Odontologia Conservadora da Faculdade de Odontologia da Universidade Federal do Rio Grande do Sul, Santana, Porto Alegre/RS, fone (51) 3308-5191, celular (51) 8177-8127 e E-mail: patricia.kopper@ufrgs.br.

Consentimento Livre e Esclarecido

Declaro que compreendi os objetivos desta pesquisa, como ela será realizada, os riscos e benefícios envolvidos e concordo em participar voluntariamente da pesquisa **"Análise da influência do laser de baixa intensidade no potencial de proliferação e diferenciação de células-tronco da polpa e da papila dental de dentes permanentes"**.

Assinatura ou impressão
Digital do responsável do voluntário: _____

Data:___/___/___

Assinatura do pesquisador: _____

Data:___/___/___

Quanto à ética dessa pesquisa poderá ser questionada ao Comitê de Ética em Pesquisa da Universidade Federal do Rio Grande do Sul (CEP/UFRGS), pelo telefone (51) 3308-3738.

ANEXO H – Termo de Assentimento do projeto in vitro

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE ODONTOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA

TERMO DE ASSENTIMENTO

Convidamos você para participar da pesquisa sobre "**Análise da influência do laser de baixa intensidade no potencial de proliferação e diferenciação de células-tronco da polpa e da papila dental de dentes permanentes**" que tem por objetivo avaliar o efeito do laser no crescimento das células-tronco da polpa dental (interior do dente), o que pode no futuro contribuir para o avanço no tratamento de várias doenças.

A sua participação terá risco mínimo, nada além daquele que o procedimento cirúrgico para extração dentária oferece, como inchaço, dor e sangramento. Você não será submetido a nenhum procedimento (extra) além da cirurgia de remoção do dente, que pode ter sido indicada porque o dente não erupcionou (dente não nasceu), por razões ortodônticas ou para prevenir reabsorção das raízes do dente vizinho. Nós utilizaremos nesta pesquisa apenas terceiros molares (*sisos*) que estejam sadios, porém você deve autorizar os pesquisadores a examinarem os seus fragmentos teciduais (dentes removidos), que seriam tirados de sua boca e desprezados após a cirurgia. Depois da remoção da polpa dental, seu dente será descartado de modo adequado (lixo biológico), seguindo os mesmos procedimentos das extrações convencionais Depois de realizar os testes para avaliar como as células do dente reagem ao laser, todo material será descartado, assim não haverá armazenamento de material recolhido. Todas as informações confidenciais serão guardadas em local seguro e somente usadas com o propósito de pesquisa, sem divulgação do seu nome.

Sua participação é voluntária, caso queira, você poderá desistir a qualquer momento, sem que isso lhe traga prejuízo ou penalidade, basta que retire o seu consentimento em participar.

A pesquisa deverá contribuir para o aumento do conhecimento no campo interdisciplinar onde princípios de engenharia e ciências biológicas são utilizados para o desenvolvimento de materiais que podem substituir tecidos e recuperar a função do ser vivo. A importância desta pesquisa, portanto, está no fato da necessidade do desenvolvimento e aprimoramento, assim como, um melhor entendimento para facilitar na escolha de tratamentos mais eficazes em regeneração de tecidos, trazendo assim, benefícios para a sociedade de um modo geral.

Você não terá nenhum gasto financeiro por qualquer procedimento executado por essa pesquisa e terá direito a reembolso (ressarcimento) de qualquer gasto comprovadamente que você tenha feito para a realização desse estudo, bem como será indenizado em caso de dano comprovadamente ocorrido por sua participação na mesma.

Você receberá uma cópia desse termo no seu endereço via correio com aviso de recebimento e qualquer dúvida a respeito da pesquisa poderá perguntar a Patrícia Maria Poli Kopper Móra, no Campus Saúde no Departamento de Odontologia Conservadora da Faculdade de Odontologia da Universidade Federal do Rio Grande do Sul, Santana, Porto Alegre/RS, fone (51) 3308-5191, celular (51) 8177-8127 e E-mail: patricia.kopper@ufrgs.br.

Consentimento Livre e Esclarecido

Declaro que compreendi os objetivos desta pesquisa, como ela será realizada, os riscos e benefícios envolvidos e concordo em participar voluntariamente da pesquisa **"Análise da influência do laser de baixa intensidade no potencial de proliferação e diferenciação de células-tronco da polpa e da papila dental de dentes permanentes"**.

Assinatura ou impressão
Digital do voluntário menor de idade: _____
Data:___/___/___
Assinatura do pesquisador: _____
Data:___/___/___

Quanto à ética dessa pesquisa poderá ser questionada ao Comitê de Ética em Pesquisa da Universidade Federal do Rio Grande do Sul (CEP/UFRGS), pelo telefone (51) 3308-3738.

ANEXO I - Termo de Doação de dentes do projeto in vitro.

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE ODONTOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA

TERMO DE DOAÇÃO DE DENTE

Prezado Sr(a),

Eu, responsável pelo menor_____, residente à _____, nº_____, aceito doar o dente _____, para o pesquisador Ivana Maria Zaccara Cunha Araújo da Faculdade de Odontologia da UFRGS, ciente que o mesmo será utilizado para a realização da pesquisa "**Análise da influência do laser de baixa intensidade no potencial de proliferação e diferenciação de células-tronco da polpa e da papila dental de dentes permanentes**". Estou ciente de que, caso eu tenha perguntas sobre este estudo e/ou sobre o órgão doado, poderei solicitar informações à professora Patrícia Maria Poli Kopper Móra (pesquisador responsável) através do telefone (51) 3308 5191. Estou ciente que caso não concorde em doar o dente para a pesquisa, não haverá qualquer interferência em meu atendimento odontológico.

_____, ___ de _____ de 201__.

_____
Assinatura do doador ou responsável

_____
Testemunha

_____
Testemunha